EFFECTS OF VARIATIONS IN DIETARY VITAMIN C ON THE PHYSICAL WELL BEING OF MANUAL WORKERS[1]

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INTRODUCTION

The primary purpose of this study was to examine carefully the early effects of total deprivation of vitamin C on the efficiency of manual workers. Information was also sought on the questions whether 75 mg. of ascorbic acid daily suffice for such workers and whether a daily supplement of ascorbic acid added to a good diet has any measurable effects in 2 months.

Our observations centered around physical fitness because of the statements in the older literature that lethargy and inefficiency are the earliest symptoms of scurvy, far antedating the onset of clinical signs (see Pijoan and Lozner, '44). This was well shown in the study of Crandon, Lund and Dill ('40) on the course and end results of scurvy induced experimentally in one subject. He began to complain of fatigue in about 60 days, but did not show clinical scurvy until considerably later.

METHODS

The experiments were conducted during the summer in Civilian Public Service Camp 32, situated at Campton, New Hampshire, in the southern part of the White Mountains at an altitude of about 2,000 feet. The days were moderately warm and the nights cool.

The twenty-four volunteer subjects were engaged in a variety of jobs associated with the camp's work schedule, which included clerical work, kitchen work, camp maintenance, farming, and work in the woods on such duties as clearing trails and road building. The range of daily caloric expenditures, depending on the subject's job, was estimated to

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be 2400 to 5000. The camp routine was disturbed as little as possible by the experiment, and the subjects carried on their usual work schedule throughout. Four subjects transferred to other projects for reasons unrelated to the experiment.

There were four groups of subjects (table 1). The "deficient group" was to demonstrate the effects of total deficiency; the "supplemented deficient group", the effects of 75 mg. of ascorbic acid daily; the "normal control group", the effects of a good normal diet; and the "supplemented normal control group", the effects of a normal diet supplemented with 75 mg. of ascorbic acid daily.

During 2 weeks of preliminary control (period 1) all groups ate the regular camp diet, which contained liberal amounts of fresh milk, eggs, meat, vegetables and fruit, provided 60 to 100 mg. of ascorbic acid daily, and was good when judged by the National Research Council standards.

During the following 8 weeks of modified diet (period 2) the two control groups continued on the usual camp diet. Each "supplemented

TABLE 1

Organization of experiment.

	DEFICIENT GROUP (8 men)		SUPPLEMENTED DEFICIENT GROUP (8 men)		NORMAL CONTROL GROUP (4 men)	SUPPLEMENTED NORMAL CONTROL GROUP (4 men)
Period 1 Preliminary control, 2 weeks	Normal diet		Normal diet		Normal diet	Normal diet
Period 2 Modified diet, 8 weeks	Deficient diet + placeboes		Deficient diet + 75 mg. ascorbic acid daily		Normal diet + placeboes	Normal diet + 75 mg. ascorbic acid daily
Period 3 Final control, 2 weeks	Subgroup A (4 men)	Subgroup B (4 men)	Subgroup A (4 men)	Subgroup B (4 men)		
	Normal diet	Normal diet + extra citrus fruit + 300 mg. ascorbic acid daily	Normal diet	Normal diet + extra citrus fruit + 300 mg. ascorbic acid daily	Normal diet + placeboes	Normal diet + 75 mg. ascorbic acid daily

normal control'' subject received at each meal one capsule containing 25 mg. of ascorbic acid, and each ''normal control'' a placebo identical to the vitamin capsule in size, shape, color, weight and taste. The ''deficient'' subjects lived on a diet devoid of vitamin C, but adequate in other respects, prepared as described below, and received at each meal a placebo. Each ''supplemented deficient'' subject lived on the deficient diet, and received at each meal a capsule containing 25 mg. of ascorbic acid.

It was found possible to destroy all of the ascorbic and dehydroascorbic acids in milk, potatoes, jams, jellies, and yellow vegetables by the addition of 10 ml. of 3% H_2O_2 per 100 gm. of foodstuff followed by 2 hours' steaming in a metal stockpot, with frequent stirring by means of copper rods. The deficient diet provided for breakfast, cereal, vitamin C free milk, coffee, egg dishes or pancakes, toast, oleomargarine and vitamin C free jam; and for lunch and supper, soup bread, oleomargarine, jam, some form of well-cooked meat, mashed potatoes, one other vegetable, a dessert, and a beverage such as coffee, tea or vitamin C free milk. The dining room was arranged so that those on the normal diet ate at one set of mess tables, and those on the deficient diet at another.

In the 2 final weeks of control observations (period 3) both normal groups continued their regime as in period 2. Both deficient groups reverted to the normal camp diet and four from each group (subgroup A) received no other vitamin C. This was to demonstrate the recuperative effects of a normal diet. Four others from each group (subgroup B) received a supplement of extra citrus fruit providing over 200 mg. of ascorbic acid daily and in addition capsules providing 300 mg. daily. This was to demonstrate the recuperative effects of massive doses of vitamin C.

In order to guard against any possibility of vitamin B deficiency, all groups including the normal controls received daily 5 gm. of yeast extract fortified with extra riboflavin.[2]

Dietary observations were made meal by meal. All articles of food were furnished in standard portions of known weight or else the subjects weighed the portion eaten. Each subject kept a record book with a page for each day. On one side of each page was recorded every article of the day's food, and on the other the day's activities hour by hour. The intakes of calories, protein, fat and ascorbic acid were estimated for each day from the tables of Bowes and Church ('42) or from our own analytical data. Any article suspected of containing significant

[2] This was Yeast Extract Type 3, kindly provided by Standard Brands, Inc. The daily dose provided approximately 5 mg. of thiamine, 5 mg. of riboflavin and 10 mg. of niacin.

amounts of ascorbic acid was analyzed by the method of Evelyn, Malloy and Rosen ('38) for ascorbic acid and that of Bessey ('38) for dehydroascorbic acid.

Observations on physical efficiency were made weekly by means of the "pack test" of fitness for hard work, as described by Darling, Johnson, Pitts, Consolazio and Robinson ('44). Scores in this test have in our experience correlated well with stamina as displayed in athletic activities, paralleled improvement in training and revealed deterioration accompanying proved inadequate diets. The Forestry project supervisors provided valuable observations on the subjects' efficiency in their jobs.

The following clinical and laboratory observations were made on each subject: (1) Complete routine physical examination during both the normal periods and at the end of the deficient period. (2) Periodic estimation of blood erythrocytes, leucocytes, hemoglobin and differential count by standard clinical methods. (3) Periodic routine qualitative examination of the urine for formed elements, albumin, sugar and acetone by standard methods. (4) Interview once a week to ascertain whether the subject was suffering from any complaint attributable to the diet. (5) Estimation of the ascorbic acid in the serum and in a 24-hour specimen of urine once a week by a modification of the titration method of Farmer and Abt ('36). (6) At the end of the deficient period, ascorbic acid tolerance tests on all of the "normal controls" and "supplemented normal controls" and on certain of the "deficient" and "supplemented deficient" subjects. After collection of a sample of blood and a timed specimen of urine, 1 gm. of ascorbic acid dissolved in 200 ml. of water was drunk. Specimens of blood and urine were collected at 3 and 6 hours after ingestion.

RESULTS

All groups maintained a reasonably good caloric balance in spite of the experimental restrictions and the somewhat unnatural diet provided for the deficient groups (table 2). Minor changes in weight and small discrepancies between calculated caloric intake and body weight are attributable to the vigorous outdoor schedule which most of the subjects undertook.

The intake of vitamin C was in general kept at the levels desired (table 2). In the final control period, the normal camp diet fell somewhat below the desired 75 mg. a day, owing to a temporary shortage of fruit.

TABLE 2

Body weight, daily caloric intake and daily vitamin C intake.

SUBJECT	JOB [3]	MEASUREMENT									
		BODY WEIGHT END OF PERIOD [1]			CALORIC INTAKE [2] PERIOD [1]			VITAMIN C INTAKE [2] PERIOD [1]			
		1	2	3	1	2	3	1	2	3	
		kg.	*kg.*	*kg.*	*cal.*	*cal.*	*cal.*	*mg.*	*mg.*	*mg.*	
		Deficient group								Subgroup A	Subgroup B
W.H	Fa	75.1	75.1	...	4590	4330	....	114	0	..	...
W.K.	K	65.7	66.3	66.5	3710	3370	3000	74	0	49	...
R.S.	Fo	77.8	75.0	76.4	3680	3050	3330	62	0	47	...
E.S.	L	62.6	62.0	62.2	2370	1800	2060	100	0	72	...
F.W.	Fo	72.9	69.5	...	3300	3000	....	73	0	..	...
N.H.	K	66.5	65.0	66.2	3490	3170	3060	100	0	..	553
L.M.	O	70.0	69.6	71.0	3140	3300	3120	76	0	..	509
M.R.	R	82.3	79.8	81.6	3620	3490	3230	85	0	..	571
Average		71.6	70.3	70.7	3490	3190	2960	86	0	56	544
		Supplemented deficient group									
W.R.	Fo	70.7	70.3	72.1	3260	3480	3130	79	75	80	...
R.S.	Fo	97.8	97.0	98.2	3570	4180	4290	78	75	82	...
R.W.	Fo	66.4	66.2	66.7	3110	2980	2850	70	75	71	...
B.W.	R	83.4	82.6	82.5	2950	2900	3170	72	75	54	...
J.C.	C	72.6	71.5	71.3	2980	3170	3750	61	75	..	646
J.L.	K	73.5	72.5	74.5	2120	2970	2440	72	75	..	531
M.P.	O	61.5	62.6	63.2	2770	3250	2960	124	75	..	514
F.S.	Fa	70.0	67.4	68.6	4840	4830	4190	98	75	..	631
Average		74.5	73.8	74.6	3200	3470	3350	82	75	72	582
		Normal control group									
W.C.	Fo	71.1	70.5	..	2830	3870	....	106	96	..	
K.K.	M	76.5	73.6	74.3	2990	2880	2860	109	95	59	
W.S.	O	69.5	66.6	68.2	3580	3380	3050	76	83	46	
R.T.	R	73.8	74.6	74.4	3040	3270	3190	52	79	58	
Average		72.7	71.3	72.3	3110	3350	3030	86	88	54	
		Supplemented normal control group									
H.H.	T	71.3	71.8	72.3	3140	3440	3300	100	151	129	
L.L.	K	77.0	75.6	77.0	3080	3100	2780	37	134	125	
T.M.	Fo	69.5	69.5	70.2	3550	3300	2970	83	172	169	
R.N.	K	71.3	69.7	...	2460	2170	....	42	134	...	
Average		72.3	71.7	73.2	3060	3000	3020	66	148	141	

[1] The experimental periods were: preliminary control, 2 weeks; modified diet, 8 weeks; and final control, 2 weeks.

[2] Calculated from weight of food consumed, analysis of diet and tables. Figures are the averages of all days in a given period.

[3] C, carpentry; Fa, farming; Fo, forestry; K, kitchen; L, laundry; M, camp maintenance; O, office work; R, road construction and repair; T, truck driving.

The physical efficiency of the subjects showed no unusual changes, and none of them complained at any time of symptoms attributable to the diet. They did their daily work efficiently and the Forestry Department supervisors detected no unusual changes in the course of the experiment. Physical fitness as measured by the "pack test" (table 3)

TABLE 3

Physical fitness for hard work. (Scores in "pack test.")

SUBJECT	PERIOD					
	Preliminary control	Experimental				Final control
	Week	Week				Week
	2	2	4	6	8	2
		Deficient group				
W.H	85	85	101	..	..	..
W.K.	74	77	83	90	83	83
R.S.	76	76	75	..	..	81
E.S.	92	100	91	91	85	95
F.W.	75	78	81	..	..	..
N.H.	78	80	81	81	88	95
L.M.	73	77	80	91	95	95
M.R.	58	58	69	78	70	66
Average change [1]		0	+5	+8	+3	+8
		Supplemented deficient group				
W.R.	87	94	106	112	111	108
R.S.	52	50	71	68	70	53
R.W.	102	94	106	94	105	90
B.W.	36	32	31	27	38	46
J.C.	92	93	98	107	104	109
J.L.	84	90	89	96	86	79
M.P.	..	..	82	80	82	79
F.S.	85	83	89	95	104	94
Average change [1]		−2	+6	+7	+10	+6
		Control group				
W.C.	71	78	72	..	..	..
K.K.	50	61	58	65	68	52
W.S.	82	81	73	85	73	91
R.T.	..	67	85	86	85	92
H.H.	105	99	110	103	102	106
L.L.	31	40	81	88	68	69
T.M.	78	86	102	87	88	99
R.N.	58	47	31	53	61	..
Average change [1]		−6	+1	+6	+3	+9

[1] These figures were obtained by setting for each individual a standard score equal to his best in the first three tests he performed, by calculating for each later score the difference from his standard, and then by averaging the individual differences for each test.

showed no significant deterioration in the deficient groups as compared with the controls. In spite of some irregularities in the data, all groups improved somewhat, and a few individuals in each group in striking fashion. These changes are most probably to be attributed to improved physical condition brought about by an active outdoor life. The small drop of the deficient group in the eighth week is paralleled by a similar drop in the control group, and both improved again to the same extent in the final control period. Irregularities in individual scores were in some cases due to changes in motivation from week to week and in others to local disabilities from minor injuries.

TABLE 4

Serum and urinary ascorbic acid. (All figures represent group averages.)

MEASUREMENT	PERIOD						
	Preliminary control	Experimental				Final control	
	Week	Week				Week	
	2	2	4	6	8	2	
	Deficient group					Subgroup	
						A	B
Serum (mg./100 ml.)	0.5	0.6	0.2	0.1	0.0	0.5	1.4
Urine (mg./24 hrs.)	16	20	10	9	9	16	683
	Supplemented deficient group						
Serum (mg./100 ml.)	0.9	1.2	0.8	0.8	0.6	1.0	1.7
Urine (mg./24 hrs.)	22	32	18	18	21	23	850
	Normal control group						
Serum (mg./100 ml.)	0.6	0.9	0.6	0.8	0.8	0.8	
Urine (mg./24 hrs.)	15	23	19	25	19	23	
	Supplemented normal control group						
Serum (mg./100 ml.)	0.7	1.0	1.1	1.1	1.1	1.1	
Urine (mg./24 hrs.)	25	41	50	65	69	93	

In contrast to the lack of change in physical effciency were the marked chemical changes (tables 4 and 5). In the "deficient group" serum ascorbic acid was zero in all but one subject by the end of the fifth week, and in his case it reacted zero by the end of the seventh week. Urinary ascorbic acid dropped to low levels. It is unlikely that the titration method would ever show zero values in urine because of reducing substances other than ascorbic acid, which probably do not disappear during a deficiency of ascorbic acid (Rosenberg, '42). Load tests revealed low body stores at the end of 4 weeks, and complete retention of the test dose by the end of the eighth week. Even after 2

weeks of normal diet, two previously deficient subjects had lower stores than the normal controls. In the "supplemented deficient group" serum and urinary levels and load tests revealed body stores about equal to those of the normal controls. The slight progressive fall in serum ascorbic acid (table 4) was entirely accounted for by one subject's (R.S.) drop to a level of 0.2 mg. per 100 ml. in 8 weeks. However, his urinary excretion and load test were as high as in other members of his

TABLE 5

Ascorbic acid tolerance tests. (All subjects received 1 gm. of ascorbic acid by mouth.)

NO. OF SUBJ.	DIETARY HISTORY BEFORE TEST	SERUM ASCORBIC ACID			EXCESS ASCORBIC ACID EXCRETED [1]	
		Before	At 3 hrs.	At 6 hrs.	In 3 hrs.	In 6 hrs.
		mg./100 ml.			*mg.*	*mg.*
		Deficient group				
2	Deficient 4 weeks	0.1	0.9	0.7	1	2
2	Deficient 8 weeks	0.0	0.9	0.8	0	0
2	Deficient 8 weeks, then normal diet 2 weeks	0.5	2.7	2.6	16	71
		Supplemented deficient group				
3	Deficient diet 8 weeks, supplemented with 75 mg. of ascorbic acid daily	0.8	2.9	2.3	84	215
		Normal control group				
4	Normal diet 10 weeks	0.8	2.1	1.9	72	262
		Supplemented normal control group				
3	Normal diet 10 weeks, supplemented with 75 mg. of ascorbic acid daily	1.2	2.6	2.2	120	486

[1] This was calculated by estimating the total excretion in the given period and by subtracting from this the excretion for an equivalent time before the test dose of ascorbic acid was ingested.

group. He was one of the heaviest of all subjects, weighing 97 kg. The "supplemented normal control group" showed the expected high serum levels, urinary levels and load tests. In the present type of oral load test a specimen of blood at 3 hours and collection of urine for the same period would have given essentially the same information as two specimens of blood in 6 hours and collection of urine for the same period.

In no group or subject was there a significant change in erythrocyte count, leukocyte count, hemoglobin or differential count throughout the experiment.

Physical examination revealed changes in only two of the twenty-four subjects in the course of the experiment. One of these (L.M.) was in the "deficient group", the other (M.P.) in the "supplemented deficient group". Subject L.M. had a congenital anomaly of the teeth in the form of three persistent primary incisors. Within 4 weeks of starting the deficient diet his serum level was zero. At the end of 8 weeks his gums, which had been normal to inspection, palpation and pressure in the preliminary control period, showed red margins, bogginess and easy bleeding on pressure. All of these changes were reversed within 10 days when his diet changed to a daily intake of over 500 mg. of ascorbic acid. Subject M.P. had an exacerbation of a previous case of Vincent's angina, which was treated with a course of bismuth. His gingivitis persisted throughout the experimental period, although it did improve slowly.

DISCUSSION

It would appear that in the case of healthy young men on a previously good diet, total deprivation of vitamin C for a period of 8 weeks has no effect on physical efficiency and produces no untoward symptoms. It may in a few individuals lead to minimal changes in the gums and does in all subjects produce marked chemical unsaturation. It would further appear that 75 mg. daily maintains ample body stores of the vitamin for 8 weeks, and that intakes of over 100 mg. daily are of no demonstrable benefit other than to increase the body stores. Our conclusions must not be extended beyond the limits of the experiment, namely 8 weeks, nor be applied to populations different from the one studied. Our data are restricted to normal, healthy young men doing their usual day's work in a temperate environment. Our subjects were deficient in ascorbic acid but they received adequate calories and ample amounts of the other essential nutrients. Their situation was perhaps not exactly comparable to that of populations subject to multiple deficiencies all at the same time.

The subject of Rietschel and Mensching ('39) was deficient for 100 days without a detectable change in efficiency, and the first symptoms of the subject of Crandon, Lund and Dill ('40) occurred not earlier than 60 days after the experimental diet was instituted. The onset of acute deficiency in healthy individuals is therefore apparently followed by clinically detectable scurvy in not less than 8 weeks and detectable inefficiency does not develop in this period. The practical implications for emergency feeding are that vitamin C is somewhat less critical than certain other factors.

SUMMARY

1. If the previous diet has been good, total deprivation of vitamin C for 2 months does not lead in manual workers to detectable deterioration in physical vigor, to inefficiency in the day's work, or to unpleasant symptoms, provided the daily diet is adequate in all nutrients other than vitamin C. Such deprivation may occasionally lead to minimal changes in the gums, and does produce severe desaturation as measured by serum and urinary levels of vitamin C and by tolerance tests.

2. When given in doses of 25 mg. 3 times daily, 75 mg. of ascorbic acid a day appear adequate to maintain or even to increase the body stores of the vitamin in a majority of men held for 2 months on a diet totally deficient in ascorbic acid.

3. Supplements of 75 mg. of ascorbic acid a day when added to a good normal diet are of no detectable benefit to manual workers over a period of 2 months with respect to general well being, physical vigor for hard work, and efficiency in the day's work. Such supplements do lead to increased stores of vitamin C as evidenced by serum and urinary levels and by tolerance tests.

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THE EFFECT OF CHAIN LENGTH OF THE DIETARY FATTY ACID UPON THE FATTY LIVER OF CHOLINE DEFICIENCY [1]

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The observation of Channon and collaborators ('36, '42) that the severity of the fatty liver in choline deficiency was influenced by the nature of the dietary fat has introduced a new factor into the fatty liver problem. The variable that was considered in their studies was the degree of unsaturation of the dietary fatty acids, and the results clearly show that the more saturated the fat of the diet, the more intense was the fatty infiltration of the liver.

In the present study the fatty acids of the diet were varied with respect to their chain length. The effect of each member of the homologous series of even-numbered, saturated fatty acids, from butyric to stearic, has been investigated. These acids have been fed, as ethyl esters, to young rats, at a level of 35% of the diet. The diet contained, in addition, 15% of casein and purified vitamin supplements. Choline was not included, however. After 2 weeks the animals were killed and their livers analyzed for fatty acids.

It will be seen (table 1) that with decreasing chain length of the dietary fatty acids from 18 to 14 carbon atoms, the mean daily weight gain of the rats increased slightly even though less food was consumed. A striking progressive increase in the fatty acid content of the livers is also apparent. When ethyl stearate was fed, normal values for liver fat were obtained. About three times as much fatty acid was recovered from the livers of the animals fed ethyl palmitate and about five times as much from the livers of the rats fed ethyl myristate. Evidence that this increasing fattiness of the livers was due in part to direct deposition of the dietary fatty acid is found in the progressive decrease in the equivalent weight of the liver fatty acids which were isolated.[3]

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[2] William J. Gies Fellow, 1943–45, University of the Philippines Fellow, 1941–45.
[3] It has been suggested by the editors that this increasing fattiness of the livers may be ascribed to the greater digestibility of the esters of the shorter fatty acids.

In these first groups of animals only one death occurred prior to the fourteenth day. When ethyl laurate was fed, however, an unexpectedly high mortality was encountered. Of the sixteen animals placed on this diet, only one survived the 14-day experimental period. All the others died between the third and the sixth days. The possibility of a toxic contaminant in the lauric acid was excluded by repetition of the experiments with rigorously purified samples. The cause of death was found to be a widespread interstitial myocarditis with necrosis of heart muscle cells. A description of this unusual lesion and studies of its prevention

TABLE 1

The effect of the chain length of the dietary fatty acid upon the quantity of liver fat.

Young male rats were placed on choline-free diets containing 35% of the ethyl esters of various fatty acids. The livers of the rats surviving 14 days were analyzed for fatty acid content.

FATTY ACID OF DIET	NO. OF RATS	MEAN DAILY FOOD CONSUMPTION	MEAN DAILY WEIGHT GAIN	DEATHS	LIVER FATTY ACIDS		MEAN EQUIVALENT WEIGHT OF LIVER FATTY ACIDS
					Mean	Spread	
		gm.	*gm.*		*% of liver*		
Stearic	4	7.5	1.2	1(14) [1]	3.2	2.3– 4.0	289
Palmitic	4	7.3	1.3	0	9.4	6.0–14.4	275
Myristic	4	5.3	1.6	0	17.2	15.5–22.4	266
Lauric	16	3.9	0.5	15(4.4)	8.0	..	261
Capric	2	6.0	1.6	0	7.2	7.1– 7.2	289
Caprylic	9	5.2	1.9	4(9.8)	4.4	2.8– 7.4	260
Caproic	2	7.0	1.2	0	7.0	6.5– 7.4	267
Butyric	2	4.0	..	2(2)	..	..	..
None	4	11.2	3.3	0	7.1	3.6–10.8	276

[1] The figures in parentheses are the average survival times, in days, of the animals which died.

are reported elsewhere (Kesten, Salcedo and Stetten, '45). It is obviously not valid to compare the quantity of liver fatty acid found in rats fed ethyl laurate with the corresponding values obtained after the feeding of other esters not merely because only one rat survived, but, more important, because these rats were suffering from a heart disease peculiar to this group. As there is no satisfactory information relating the state of heart failure to the quantity of liver fat, such a comparison would only serve to confuse the picture.

With fatty acids shorter than lauric acid no severe fatty livers were obtained. Several deaths occurred in the group of rats receiving ethyl caprylate. The chief pathological finding in this group was renal hemorrhage and necrosis, similar in distribution to that described pre-

viously as occurring in choline deficiency (Engel and Salmon, '41). Curiously, these kidney lesions were observed in none of the animals receiving other fatty supplements except in one that had been fed ethyl laurate.

The feeding of ethyl butyrate resulted in death within 2 days. At autopsy, the intestinal tracts of these animals were found to be full of blood, and microscopic examination revealed acute gastric ulceration as the site of hemorrhage.

To a control group of rats a diet devoid of fatty supplement was fed, the carbohydrate of the diet being correspondingly increased. These animals consumed more food and gained more in weight than did any of the other groups. They developed mild fatty livers and the equivalent weight of the liver fatty acids was almost the same as when palmitic ester was fed. This is in accord with the view that it is predominantly the 16-carbon fatty acid that is synthesized by rats on a high carbohydrate diet (Longenecker, '39).

Our results, considered together with those of Channon et al., ('36, '42), show that the response that may be expected to a diet devoid of lipotropic methyl will be critically dependent upon the nature of the fat in the diet. The resulting fattiness of the liver will be influenced not only by the degree of unsaturation of the dietary fat but also by the molecular weights of the constituent fatty acids. It is possible that some of the discrepancies observed by several workers in the fatty liver problem are due to unrecognized variations in the composition of the dietary fat.

EXPERIMENTAL

Samples of stearic, palmitic, myristic and lauric acids [4] were esterified with ethyl alcohol in the usual fashion and the products distilled in vacuo through a Vigreux column. Commercial samples of lauric acid and ethyl laurate,[5] were also used. One portion of lauric acid was subjected to careful preliminary purification by solution in warm conc. H_2SO_4, precipitation by dilution with water, followed by repeated recrystallization from aqueous acetone. The lower fatty acids were all commercial products.[6] The purity of each fatty acid was controlled by determination of its titration equivalent weight and iodine number.

The diet used contained 35% of fatty acid ethyl ester, 15% of casein,[7] 44% of glucose monohydrate, 4% of salt mixture no. 2, U.S.P. XII and

[4] The authors wish to express their gratitude to Mr. H. Sherman of the Massachusetts Institute of Technology for his gift of these samples.

[5] Samples of lauric acid and ethyl laurate were purchased from the Eastman Kodak Company.

[6] Lower fatty acids were purchased from the Eastman Kodak Company.

[7] Labco, vitamin-free.

2% of roughage.[8] To each milligram of this diet were added 6.25 mg. of thiamine hydrochloride, 5 mg. each of riboflavin and pyridoxine, and 25 mg. each of niacin and calcium pantothenate. The fat-soluble vitamins were given by mouth to each animal in the following doses: 1,700 units of vitamin A and 200 units of vitamin D each week, and 0.2 ml. of linseed oil every 10 days. In the diet of that group of rats that received no fatty acid ethyl ester, the carbohydrate fraction was increased from 44 to 79%.

Male rats of the Sherman strain, about 5 weeks old and weighing 55–60 gm. were used throughout. The animals which survived the 14-day period were killed by asphyxiation with N_2. Their livers were individually digested with alkali and fatty acids isolated in the usual fashion (Stetten and Schoenheimer, '40). The quantities of fatty acid tabulated (table 1) are averages of individual determinations. The liver fatty acids of each group were pooled and their mean equivalent weights determined by titration with 0.1 N alkali in 50% ethanol.

SUMMARY

Each member of the homologous series of even-numbered fatty acids, from butyric to stearic, has been fed as ethyl ester, at a 35% level in the diet, to young male rats receiving no choline.

The degree of fatty liver was found to increase markedly as the chain length of the dietary fatty acid decreased successively from 18- to 16- to 14-carbon atoms. No severe fatty livers were encountered when the ethyl esters of fatty acids of less than 12-carbon atoms were fed.

Renal hemorrhage and necrosis occurred in several of the rats fed ethyl caprylate and a fatal myocarditis was encountered when ethyl laurate was fed.

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[8] Celluration.

FATAL MYOCARDITIS IN CHOLINE DEFICIENT RATS FED ETHYL LAURATE [1]

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FOUR FIGURES

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In the course of a study of the relationship of chain length of dietary fatty acids to fatty liver (Stetten and Salcedo,'45), an unexpected mortality was encountered when rats on a choline-free diet were fed the ethyl ester of lauric acid. It was observed that of a total of sixteen rats on a synthetic diet containing 35% of ethyl laurate, all but one died between the third and sixth days. Prior to death these animals exhibited no untoward symptoms although they consumed less food than did rats receiving the ethyl esters of homologous fatty acids. Animals were observed to be normally active within 2 hours prior to death.

That congestive heart failure was the cause of death was clear from the pathological findings. The hearts were small, and the ventricles pale and in systole. Effusions in the body cavities were occasionally noted.

Histological examination revealed the cause of the heart failure to be a widespread interstitial myocarditis associated usually with necrosis of isolated muscle cells. In some cases the myocardium of all the chambers was involved in a patchy fashion; in others, only that of the ventricles. The subendocardial regions seemed especially susceptible (figs. 1 and 2) but the conduction system was generally not strikingly involved nor were thrombi formed on the overlying endocardium.

The interstitial tissue was spread apart and infiltrated by wandering cells that included large mononuclears, lymphocytes and neutrophilic polymorphonuclears, but macrophages and lymphocytes predominated (fig. 3). A few cells resembling plasma cells were also present. The cytoplasm of the large mononuclear wandering cells was devoid of phagocytized material. Fibrin was not present, but a mild degree of

[1] This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.
[2] William J. Gies Fellow, 1943–45; University of the Philippines Fellow, 1941–45.

edema was suggested by the presence of a little fine eosinophilic granular material about the wandering cells. Giant cells were not found.

The extent of visible damage to the heart muscle cells was variable. In the more severe cases a considerable number of swollen necrotic cells were found, singly, or in small groups of a few cells (fig. 4). The cytoplasm was altered in a segmental manner, with loss of cross-striations, and pallor of the sarcoplasm. In the less severely affected hearts, histologically demonstrable damage to the muscle cells was not always seen, although the interstitial inflammatory reaction was present, as indicated by small groups of wandering cells at many points. No evidence of regeneration of muscle or of proliferation of connective tissue was observed in any of the animals, nor could any undue accumulation of cardiac fat be demonstrated histologically.

Except for the fatty livers, the other organs of the rats exhibited no microscopic alterations beyond congestion, this being sometimes quite marked in the liver.

It should be pointed out that these cardiac changes, in our series, appear to be specificially related to the ingestion of lauric acid. We have fed, as ethyl ester, each member of the homologous series of even-numbered saturated fatty acids to rats and in only one animal receiving a fatty acid other than lauric were myocardial inflammatory changes observed. This rat, fed ethyl stearate, died on the fourteenth day of feeding, and this isolated case may well be unrelated etiologically to the disease which results from lauric acid feeding.

In view of the inflammatory nature of the cardiac lesion, bacterological studies have been carried out. Heart tissue was taken for culture from three rats by Dr. H. Rose of the Department of Medicine. These rats had been sacrificed 4, 5 and 6 days, respectively, after being placed on the ethyl laurate diet. The cultures showed no growth on aerobic or anaerobic media. Blood smears have also been examined for parasites but none was found.

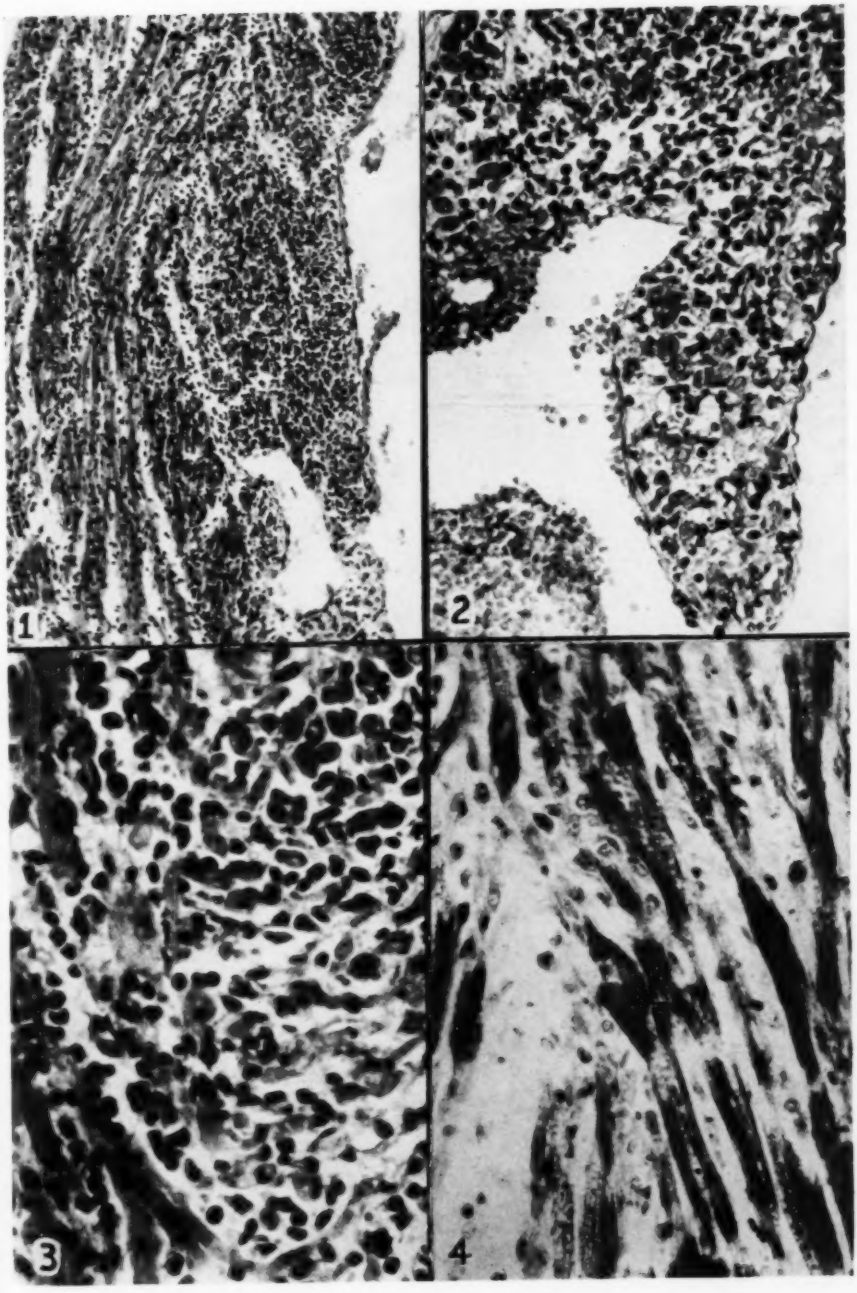
In a further study of the myocardial lesion, the concentration of ethyl laurate in the diet has been varied (table 1). Whereas a 40% level proved uniformly fatal, a 25% level was tolerated. Even in these

Fig. 1 Rat 44–44. Interstitial myocarditis in wall of right ventricle, especially near endocardial surface (× 120). Ethyl laurate 35%. Death on fourth day.

Fig. 2 Rat 44–19. Subendocardial and interstitial round cell infiltration. (× 300). Purified ethyl laurate 35%. Death on fourth day.

Fig. 3 Rat 44–44. Acute interstitial myocarditis with fragmentation of muscle cellls in wall of right ventricle (× 460).

Fig. 4 Rat 44–44. Degeneration and necrosis of myocardial cells. (Mallory's phosphotungstic acid hematoxylin stain. × 460).



Figures 1 to 4

animals, however, histological evidence of diffuse interstitial myocarditis was found, though to a much milder degree than in the rats fed 35% of ethyl laurate. The addition of 0.5% of *l*-cystine to a diet containing 25% of ethyl laurate appeared to aggravate the pathological process in the heart slightly. The degree of fatty liver in these rats was more marked.

TABLE 1

Effect of various supplements upon rats receiving ethyl laurate.

Ethyl laurate and various supplements were incorporated into the diets of young male rats in the concentrations indicated. The livers of the rats surviving 14 days were analyzed for fatty acids.

ETHYL LAURATE	SUPPLEMENT	NO. OF RATS	MEAN DAILY FOOD CONSUMPTION	MEAN DAILY WEIGHT GAIN	DEATHS	LIVER FATTY ACIDS	MEAN EQUIVALENT WEIGHT OF LIVER FATTY ACIDS
% of diet	*% of diet*		*gm.*	*gm.*		*% of liver*	
35	..	16	3.9	0.5	15(4.4) [1]	8.0	261
40	..	4	..	..	4(5.5) [1]	..	..
25	..	2	5.5	0.9	0	15.0	254
25	*l*-Cystine, 0.5	2	4.6	1.3	0	20.6	257
35	Choline chloride, 0.5	4	6.0	1.7	0	4.0	256
35	Inositol, 0.5	2	2.6	−1.2	2(5.5) [1]	..	..
35	Betaine hydrochloride, 1.25	2	3.5	+0.6	2(6.5) [1]	..	..
35	Betaine hydrochloride, 2.5	2	5.3	2.0	0	3.8	256
35	*dl*-Methionine, 1.25	2	3.6	−0.2	1(4) [1]	7.8	259
35	*dl*-Methionine, 2.5	2	4.5	+1.2	0	2.8	260
35	K_2HPO_4, 3.5 [2]	6	2.9	−0.96	6(4.3) [1]	..	..

[1] The figures in parentheses are the average survival times, in days, of the animals which died.
[2] The stock diet contains 0.96% K_2HPO_4.

A few studies of the prevention of the cardiac lesion have been completed. The addition of 0.5% of choline chloride to a diet containing 35% of ethyl laurate completely prevented the appearance of myocardial changes. The same concentration of inositol was devoid of any prophylactic action. Betaine hydrochloride, incorporated in the diet at a level of 1.25% did not prevent the disease, but at twice this concention, it was completely effective. Likewise, *dl*-methionine was only partially prophylactic at a level of 1.25%, but at 2.5% prevented the appearance of any cardiac changes. The expense of the purified fatty

acids precluded the use of large series of animals. The prevention of the cardiac lesion by these "lipotropic agents" was sufficiently dramatic in the small groups of animals used, however, to warrant its inclusion in the present paper.

In their description of the pathological findings in rats on choline deficient diets. Engel and Salmon ('41) incidentally mention myocardial changes. As these authors were not primarily concerned with variations in dietary fat, it is difficult to say whether they were observing the same disease as we have described. In the lesions that they observed, myocardial hemorrhage is prominent, a phenomenon not encountered in our series.

Follis, Orent-Keiles and McCollum ('42) have described cardiac changes, very simliar histologically to those encountered in the present experiments, which occurred when diets extremely poor in potassium were fed. Their disease was characterized by survival for 6–47 weeks, by complete prevention when the level of K in the diet was raised to 0.44%, and by a decrease of some 30% in the K content of the heart. Our rats normally received 0.43% of K in their diets. Further increase of the dietary K to a value of 2.0% did not interfere with the development of myocarditis or the fatal income. The possibility that our ethyl laurate diets caused a depletion of K in the heart muscle cells has been ruled out by potassium analyses on the hearts of rats which had been fed the disease-producing diet for 4 days. The addition to the diet of choline, which has been shown to prevent the disease, did not significantly alter the myocardial K content (table 2).

A cardiac failure incident to an acute interstitial myocarditis, fatal in about 4–5 days, may thus be provoked in young rats on a diet poor in lipotropic agents, if 35% or more of the diet is supplied as ethyl laurate. The disease, quite specifically related to the 12-carbon fatty acid, appears to be due neither to a toxic contaminant nor to a bacterial or parasitic agent. It must therefore be supposed to be related to some property of lauric acid itself. It is perhaps worth noting that certain other biological peculiarities of lauric acid have been reported in the literature (Siegler and Popenoe, '24; Ozaki, '27; Powell, '30).

EXPERIMENTAL

The ethyl laurate used in these experiments was prepared and purified as described in the preceding paper. When it was fed at a 35% level, the remainder of the basal diet was the same as that previously described. In the experiments in which ethyl laurate was fed at a 25% level, the carbohydrate of the diet was increased to 54%. To one group

of animals ethyl laurate was fed at a 40% level. In this case, the casein was cut to 10% of the diet. The vitamin supplements were given in all cases as mentioned in the previous report.

Additional supplements of choline chloride, meso-inositol, l-cystine, dl-methionine, betaine hydrochloride and K_2HPO_4 were added to diets in concentrations indicated in table 1.

TABLE 2

Potassium content of hearts and kidneys of rats fed ethyl laurate.

Two groups of 3 rats each were kept for 4 days on diets containing 35% of ethyl laurate. Choline chloride, 0.5%, was incorporated in the diet of the control group. Pooled hearts and kidneys from each group were analyzed for K.

		FRESH WEIGHT	DRY WEIGHT	K	K	K
		mg.	*mg.*	*mg.*	*% of dry weight*	*% of ash*
No choline added	Hearts	649	133	2.43	1.82	41
	Kidneys	1852	416	4.87	1.17	21
Choline added	Hearts	631	131	2.56	1.95	43
	Kidneys	1667	376	4.31	1.15	21

Two groups of three rats each were placed on the 35% ethyl laurate diet. To the diet of one group, 0.5% of choline chloride was added. After 4 days, the rats were killed, and the hearts and kidneys then removed. The pooled hearts and the pooled kidneys from the rats of each group were dried to constant weight of 100°C., ashed (Stolte, '11), and the ash in each case analyzed for K (Shohl and Bennett, '28) (table 2).

All the remaining operations were carried out as previously described (Stetten and Salcedo, '45).

SUMMARY

The feeding of ethyl laurate, at a 35–40% level in the diet, to young rats receiving no choline resulted in death from heart failure in from 3 to 6 days. The pathological process was an acute diffuse interstitial myocarditis. It was preventable by the adequate administration of choline, betaine or methionine. The disease did not occur in rats fed the ethyl esters of other fatty acids and was not fatal when the quantity of ethyl laurate was reduced to 25% of the diet. Though histologically similar, the disease is apparently not identical with the myocarditis of potassium deficiency.

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OBSERVATIONS ON RIBOFLAVIN EXCRETION BY THE ADULT MALE [1]

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ONE FIGURE

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If the requirement of the human adult for riboflavin is as high as suggested by the Food and Nutrition Board of the National Research Council ('43), recent surveys (Bing, '43) indicate that riboflavin deficiency is one of the commonest inadequacies in the diet of American laborers. Investigation of the quantitative aspects of this riboflavin requirement was undertaken as a part of the study begun in March, 1942, of the nutritional requirements of adult males (Kyhos et al., '44). Subjects were chosen from male inmates of the Wisconsin State Prison at Waupun; all were in good health, doing moderate to heavy work in prison industries, and had been inmates at least a year. Their co-operation was voluntary. To get data on the riboflavin requirement, it was our intention to determine the basic level of riboflavin excretion and the effect of supplements on riboflavin excretion; to note sources of riboflavin in the diet; and to conduct periodic physical examinations to observe any clinical signs that could be correlated with variations in intake and excretion. These observations extended over a period of 2 years.

Physical examinations were limited to inspection of the sclerae, skin about the eyes, nose and mouth, the lips, tongue and gums. A portable slit lamp providing about 4 diopters magnification was used.

DIET

Details of the prison diet have been given in a previous report (Kyhos et al., '44). It is high in carbohydrates and muscle meats, low in fresh fruits and fresh vegetables. Butter is served once a day, eggs and cheese each twice a week. Milk is used in cooking various foods,

[1] This project has been made possible by special grants from the Wisconsin Alumni Research Foundation at its inception, and has been supported chiefly by the assistance of the Nutrition Foundation, in a grant to one of us (E.L.S.).

and is mixed with the coffee before serving. The recipe calls for 10 pounds cream and 40 pounds whole milk in 100 gallons of coffee, i.e., 1 part in 16. Three times a week cereal and milk are given at breakfast, and the men may drink as much milk as they want at this time. Liver is served at irregular intervals, never oftener than once a month.

It was not practicable to keep the subjects on a measured diet, or to determine the amount of riboflavin in the food as actually served and consumed. From inspection of the menus it appeared that milk would be the chief source of riboflavin, and that all the men had the opportunity to drink 2 or 3 cups of milk three times a week. Some were given extra milk for various reasons, and some used their earnings to buy extra fruit, candy, peanuts and vitamin preparations.

METHODS

The microbiological method of Snell and Strong ('39) was used. All analyses were done by the same operator. Excretion was determined by assay of 24-hour urine samples, collection beginning at 6 A. M. For protection against light, alkaline urine, and bacterial decomposition, dark brown glass bottles were used, containing as preservative 10 ml. glacial acetic acid, 1 ml. toluene, and 1 ml. chloroform. Bottles were kept cool in a dark container, and taken by truck the next morning to the laboratory at Madison, 55 miles away. On arrival, samples were checked to insure acidity, and refrigerated under toluene. A total of 360 24-hour samples was examined. Samples were assayed within 1–3 days after collection. On 180 of the samples, analysis was repeated within 3 weeks, and no significant variation was noted in riboflavin content: 120 showed less than 10% variation; of the remaining 60 none varied more than the limit of 20% ascribed to the method by the authors.

RESULTS

Preliminary survey

Single samples from 68 men ranged in riboflavin content from 0.05 to 2.4 mg. No correlation appeared between this variation and the ages and weights of the men. Ages ranged from 19 to 59, weights from 130 to 218 lbs. It will be readily understood that in such an institution detailed investigation cannot be undertaken with all types of inmates. On the other hand, institutional regulations prevent indiscriminate access to food. Purchases and gifts are each limited to once a month and are a matter of record. Extra or special foods from the prison are obtained on medical recommendation. Intelligence and cooperation on the part

of the men also assisted in obtaining definite and reliable information about the food habits of 43 individuals. In table 1 a summary of these observations is presented which emphasizes the correlation between riboflavin excretion and the intake of materials rich in riboflavin: milk, liver, peanuts and vitamin preparations.

TABLE 1

Ranges in riboflavin excretion shown in single 24-hour specimens from 68 men.

TOTAL NO. OF MEN	24-HOUR EXCRETION	FOOD HABITS OF 43 MEN
	mg.	
25	0.05–0.2	7 drank no milk; 7 drank less than 3 cups per week.
27	0.2 –0.5	16 drank 9–10 cups of milk per week (regular diet).
16	0.5 –2.4	13 drank extra milk, ate peanuts, or took vitamin tablets.

Intensive study preliminary to supplementation

Of the 43 men with known food habits, we were able to continue the study of 29, who in different groups furnished 24-hour urine samples once weekly over a period of 15 months. Sunday was selected as the day of collection because of convenience for the prison staff and inmates. The menus for Saturday and Sunday were standard, including cheese once, meat twice daily, and eggs and milk for Sunday breakfast. Urine collection on Sunday and on the following Monday by 16 men showed substantial agreement in each case. One man on the regular diet showed an average excretion in 15 samples of 0.28 mg. He collected 6 consecutive daily samples, with riboflavin content as follows: Sunday, 0.26 mg.; Monday, 0.27 mg.; Tuesday, 0.18 mg.; Wednesday, 0.22 mg.; Thursday, 0.38 mg.; Friday, 0.46 mg.; and on these last 2 days holiday meals including chicken with liver dressing were served. We felt therefore that samples collected on Sunday were fairly representative of the usual excretion.

The 29 men were classified in three groups. Group A consisted of 16 men eating the regular diet which included 9–10 cups of milk per week. In group B there were 5 men who drank no milk and used none on cereal. Group C was made up of 9 men who ate the regular diet plus extra milk, peanuts or vitamin tablets. A total of 108 weekly urine samples were collected by the group of 29. While occasional individual variations occurred from 1 week to another, the average excretion for each man, and for each diet group, showed a correlation with the intake of riboflavin (table 2).

In group A, the individual average excretion (table 2) ranged from 0.2 mg. to 0.47 mg. with a group average of 0.3 mg. Average excretion per month ranged from 0.21 to 0.35, with no seasonal variation. General daily average was 0.3 mg. Again, no correlation with weight or age could be found. In group B, men who drank no milk, the average excretion was 0.1 mg., with a range of 0.05 to 0.12. In group C, excretion varied with the type of extra nourishment taken. That of men taking vitamin tablets was always above 1.0 mg. Men taking an extra pint of milk daily excreted slightly less than 1.0 mg. Those eating peanuts excreted about 0.6 mg. The riboflavin content of these materials, taken from food charts published by the American Medical Association ('42) and from

TABLE 2

24-hour excretion of riboflavin in special diet groups.

GROUP	A		B			C			EXTRA SOURCE OF RIBOFLAVIN
No. of men	Av. excr.	Total N	No. of men	Av. excr.	Total N	No. of men	Av. excr.	Total N	
	mg.	*gm.*		*mg.*	*gm.*		*mg.*	*gm.*	
8	0.2–0.3	10.3–14.5	5	0.05–0.12	7.4–12.4	2	1.3, 1.5		Vitamin preparation A
6	0.3–0.4	8.5–15.5				1	1.4		Vitamin preparation B
2	0.4–0.47	13.8				3	0.9, 0.7, 0.5	7.2–14.2	½–1 pt. milk
						2	0.6, 0.56	9.5–11.5	Peanuts

the manufacturer's claims of potency for vitamin preparations are as follows: peanuts 1.76 mg./lb., milk 0.96 mg./pint, liver 3.40 mg./4 oz., vitamin preparation A 2.00 mg./1 tablet, and vitamin preparation B 2.00 mg./3 tablets.

The total nitrogen excretion data in table 2 give an idea of the level of nutrition and especially of the meat intake in these subjects. The range of 7.2 to 15.5 gm. nitrogen excretion indicates that nitrogen equilibrium might well be expected, since these men have access to as much food as they desire at meal time, and were in approximate weight equilibrium. No correlation could be established between the nitrogen excretion and riboflavin in the 24-hour urine samples, probably because

muscle meats and cereal products were the chief sources of nitrogen in the diet. There was no tendency to increased riboflavin excretion with low nitrogen levels, as might be expected from the observations of Sarett and Perlzweig ('43) on rats. However their data indicated that in order to obtain riboflavin deposit in the liver the rats must be depositing protein. Thus there is no reason to expect that a moderately low nitrogen level, especially in equilibrium conditions, would tend to increase riboflavin loss.

Results of physical examinations

In group A, using the ordinary prison diet, 14 of the 16 men showed grossly visible corneal injection, usually accompanied by various sizes of yellowish plaques of tissue on one or both sides of the pupil. In this group 5 had tongues with distinct furrows, of varying pattern. But only one man had scars at the canthi of the lips, suggesting former active lesions, and there were none with lesions which could be described as cheilosis.

In group C, of the 8 men who used supplements of various sorts, 4 had similar corneal lesions, 2 had furrowed tongues and a third had an atrophic tongue. It was evident that 3 had scars from old cheilotic lesions, and that a fourth member of the group had suggestive lesions of current disturbance possibly to be called cheilitis.

By contrast the observations of the 5 men in group B, who by taste had been avoiding milk for 2 or more years, showed that only one had the corneal lesions described above. None of these men had a furrowed tongue. Search for the lesions at the canthi of the lips, about the nasolabial folds, or about the eyes led to discovery of only one man with questionable cheilitis at the lips. This was in the individual with the lowest excretion of riboflavin, averaging 50 μg. daily.

Effect of supplements

Although no clinical signs attributable with certainty to ariboflavinosis had been observed, the plan of giving riboflavin supplementation was carried out. Excretion due to supplements of natural sources of riboflavin was compared with that following administration of synthetic riboflavin. Supplements were given for 3 to 6 weeks to groups of 5–6 men each, with collection of 180 weekly urine samples as before. Due to difficulties of various sorts the final groups who completed the studies were composed of 2 to 5 men each. The milk supplement was given between meals. Yeast tablets and tablets of synthetic riboflavin

were given after meals, to avoid too rapid absorption and excretion. Each yeast tablet contained 0.4 gm. of dried unfermentable brewer's yeast fortified with a natural riboflavin concentrate from processed corn, furnishing about 0.085 mg. of riboflavin per tablet.[2] Synthetic riboflavin[3] was used in tablets of 0.5, 1.0 and 2.0 mg. content.

Table 3 summarizes the effects of supplementation on the excretion of men in group A (9–10 cups of milk per week). Average excretion was increased 0.2 to 0.28 mg. by supplements of 0.5 mg.; it was increased 0.7 to 0.8 mg. by supplements of 1.0 mg. in the form of milk and yeast,

TABLE 3

Retention of riboflavin supplements.

NO. OF SUBJECTS	DAILY SUPPLEMENTS	AVERAGE EXCRETION		SUPPLEMENT RETAINED
		Before	After	
		mg.	*mg.*	*mg.*
4	Milk — 1 cup (0.48 mg.)	0.35	0.63	0.20
4	Milk — 2 cups (0.96 mg.)	0.35	1.15	0.16
3	6 yeast tablets (0.5 mg. riboflavin)	0.30	0.50	0.30
2	12 yeast tablets (1.0 mg. riboflavin)	0.30	1.00	0.30
3	1.0 mg. synthetic riboflavin	0.30	0.50	0.80
5	2.0 mg. synthetic riboflavin	0.30	1.58	0.72

and increased 0.2 mg. by 1.0 mg. of synthetic riboflavin. To illustrate the consistency of the increase: one subject with an average basic excretion of 0.28 mg. excreted 0.50, 0.55, 0.47 and 0.50 mg. in 4 consecutive daily 24-hour samples while taking 1.0 mg. of synthetic riboflavin daily. During the administration of 6 yeast tablets daily, a meal of liver resulted in excretions ranging from 0.87 to 1.34 mg.; these readings were excluded from the averages.

Table 4 gives data on supplementation of group B, men on the regular diet who drank no milk. Their basic excretion (average 0.1 mg.) was raised 0.15 to 0.2 mg. by addition of 0.5 mg. of synthetic riboflavin; it was raised 0.35 to 0.6 mg. by 1.0 mg. daily.

Men in group C, with a high basic excretion due to extra intake of milk responded to supplementation by a further rise in excretion (not

[2] Fleischmann

[3] Merck.

shown in tables). For example, a group with an average excretion of 0.7 mg. excreted over 1.0 mg. daily while taking 6 yeast tablets (0.5 mg. riboflavin) daily; a meal of liver during this period produced excretions ranging from 1.36 to 2.84 mg. the following day.

Probably the significance of these data lies in the amount of supplement retained rather than in the amount excreted. From the data in table 3 it is seen that when milk supplements were used, and assuming the milk content of riboflavin to be 0.96 mg. per pint (Food Charts, '42), the retention was about 41% with one-half pint, but only 17% of the supplement when a pint was taken. This retention is significantly less than that reported by Gardner et al. ('43) on a milk diet yielding 7.0 mg. riboflavin. Using yeast supplements of comparable riboflavin content, the retention was 60% on 0.5 mg. intake, but only

TABLE 4

Group B — Riboflavin excretion. Before and after supplements of synthetic riboflavin.

SUBJECT NO.	BEFORE SUPPLEMENT		AFTER SUPPLEMENT			
			0.5 mg. supplement		1.0 mg. supplement	
	"Fasting" hour excretion	24-hour excretion	24-hour excretion	Retained	24-hour excretion	Retained
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
22	0.	0.05	0.20	0.35	0.40	0.65
20	0.003	0.10	0.25	0.35	0.50	0.60
23	0.005	0.10	0.25	0.35	0.60	0.50
21	0.007	0.10	0.30	0.30	0.70	0.40
Average		0.10	0.25	0.33	0.55	0.55

30% on 1.0 mg. When pure riboflavin was given in tablet form, retention of 80% was observed on 1.0 mg., dropping to 36% when the dose was doubled. Data taken from table 4, where the men were on a lower riboflavin intake, indicate that after supplementation with pure riboflavin in 0.5 mg. tablet doses retention averaged 66%, and that when the dose was increased to 1.0 mg. the retention was still at 55%. By the criteria of basic urinary excretion as well as the type of diet used these men were more apt to be unsaturated than any other men we have studied. Yet their retention of riboflavin supplements was not strikingly greater than that of the others on a higher intake.

These same relations are shown graphically in three curves (fig. 1). The total excretion is plotted as ordinates, the supplemental intake as abscissae. It will be noted that the excretion rises faster than the rate of increase in amount of supplement. This relation was true in

every individual case where the subject received two different doses of the same supplement. Such a relationship between intake and excretion makes it probable that riboflavin is a non-threshold substance, excreted by the kidneys even when the intake is very low. The curves connecting the points on these three typical subjects have been extrapolated to the intersection with the base line. This procedure should serve to indicate an approximate intake on the unsupplemented diet, by the distance to the left of the ordinate axis. The two men on the ordinary prison diet appear to be securing about 1.5 mg. riboflavin.

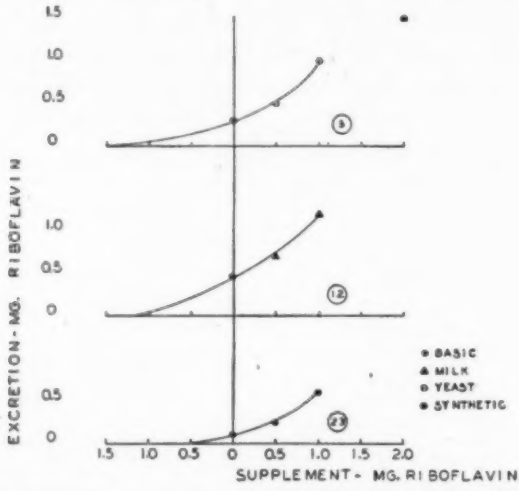

Fig. 1 Rate of increase in riboflavin excretion with diet supplementation. Subject 5, on ordinary prison diet, supplemented with yeast and with synthetic riboflavin. Subject 12, on ordinary prison diet, supplemented with milk. Subject 23, usually avoiding milk, supplemented with synthetic riboflavin.

The one who avoided milk was getting about 0.5 mg. The data from the other members of this group suggest intakes of the same order of magnitude. We are therefore inclined to believe that these men have been subsisting for a period of 2 or more years, as they asserted, on a diet which provides this very small amount of riboflavin. Yet we were unable to detect in them any of the physical findings supposedly characteristic of riboflavin deficiency. Furthermore, after they had been receiving the supplements for periods of several weeks, they gave no consistent reports of any subjective improvement.

In table 4 the riboflavin excretions in 1-hour urine samples, taken just before breakfast, are compared with the excretions in the succeed-

ing 24 hours when no riboflavin supplement was administered. Considering the great errors introduced when the concentrations are so low, the agreement is fair. We are not impressed with the usefulness of such single hour specimens (Holt, '43) for this type of study.

On 180 of the 24-hour urine samples, determination of pantothenic acid was done by the microbiological method of Strong, Feeney and Earle ('41). Individual variations were marked at all seasons. Most of the values were in or near the accepted normal range of 2–5 mg. (Gordon, '42). No correlation was seen with food intake, with riboflavin excretion, or with changes in riboflavin intake. The extreme range of values was 1.1 to 6.6 mg. Pantothenic acid determinations were not done during periods when yeast was administered.

DISCUSSION

The physical findings in our subjects fail to convince us of any correlation between riboflavin intake and such lesions as cheilosis, changes in naso-labial skin, or chronic lesions of the sclerae. In fact such lesions were far more common among men who were on fair or supplemented diets than among those whose long-standing habitual avoidance of milk kept them on the lowest riboflavin intakes we have observed. We consequently must question cheilosis, chronic lesions at the naso-labial folds, or thickened and vascularized patches in the sclerae as being evidences of long continued riboflavin deficiency.

We have no accurate measure of the total riboflavin intake of our subjects. The excretion of riboflavin was demonstrably increased by milk, by other foods such as liver or peanuts, by the use of pure riboflavin in tablet form, or by yeast tablets. The data in tables 3 and 4 indicate that of the various supplements the retention was from 17 to 80%. A variable state of saturation does not appear to be a sufficient explanation for this wide fluctuation. The data are not isolated observations, but are based on averages of excretion at weekly intervals. There was no distinct increase or decrease of excretion in such a series of weekly averages to suggest a marked change in the state of saturation during the period of weeks while a given supplement was being used.

The excretion of 50 to 100 μg. riboflavin by the men in our group B is of the same order of magnitude as that reported by Keys et al. ('44) for their subjects who received 0.82 to 0.90 mg. daily. This circumstance adds to our conviction that our subjects were receiving not much more than 0.5 mg. daily in their conventional intake. Keys et al. show retentions of supplemental administered riboflavin varying from 38 to 90%.

If the intake of much less than 1.0 mg. daily of riboflavin for such long periods as 2 years fails to induce cheilosis or the other lesions formerly attributed to deficiency of this factor, we are led to question the nature of objective data for the need of riboflavin by the adult human. The report of Williams et al. ('43) deals with subjects whose dietary intake was similar to ours except for better supplementation with other vitamins. Their only evidence of riboflavin deficiency was decreased excretion of test doses, i.e., of tissue desaturation. In our opinion this does not demonstrate any need for the vitamin. Doubt about the validity of saturation tests has been expressed by Axelrod et al. ('41). It would be surprising if there were no requirement for a vitamin which is a constituent of an important enzyme system. We believe riboflavin is a vitamin for the human. The urgent need is for evidences from physical examination or from biochemical studies which will demonstrate this need and allow detection of subjects with deficiency. When this end has been achieved the magnitude of the need can be studied.

SUMMARY

Studies made on a group of adult male prisoners showed that after ingestion of riboflavin supplements in the form of tablets or foods rich in vitamins there was an increase of riboflavin excreted in the urine. The retention of riboflavin from such supplements varied from 17 to 80% with no apparent reason for the wide fluctuations. No physical findings were seen to indicate riboflavin deficiency in those men who for 2 years or more must have been eating not much over 0.5 mg. daily, and who were excreting 0.05 to 0.12 mg. riboflavin in the 24-hour urine. It is felt that satisfactory criteria of need for riboflavin by the human adult are still lacking.

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NIACIN (NICOTINIC ACID), AN ESSENTIAL GROWTH FACTOR FOR RABBITS FED A PURIFIED DIET

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FIVE FIGURES

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Hogan and Ritchie in 1934 reported successfully raising rabbits on simplified diets. Their most simplified successful diet consisted of commercial casein 20%; dextrinized cornstarch 30%; milkfat 13%; cod liver oil 1%, wheat germ oil 1%, dried yeast 15%; salts 5% and cellophane 15%. In 1942 Hogan and Hamilton reported that guinea pigs and rabbits grew at the normal rate on simplified rations which contained dried yeast as the source of all water-soluble vitamins. When the water-soluble vitamins were supplied as pure compounds then available, the mortality was high and the rate of growth was subnormal.

Swaminathan('42) reported that "rabbits do not require an extraneous source of nicotinic acid and that they were able to synthesize it." His diet consisted of extracted oats 46%, sugar 10%, purified casein 10%, whole cow's milk 31% and salt mixture 3%. This basal diet was shown by analysis to contain an average of from 70 to 82 μg. of nicotinic acid per 100 gm. Five rabbits fed this diet gained an average of 235 gm. in 10 weeks. Five animals that were fed the diet plus 3 mg. of nicotinic acid gained an average of only 175 gm. in a similar period.

Under our experimental conditions reported below nearly normal growth has been maintained in rabbits for a limited period, at least, on our purified diet supplemented only with pure vitamin preparations. Rabbits fed this purified ration require the addition of niacin (nicotinic acid) for satisfactory growth.

EXPERIMENTAL

White rabbits from a strain raised at the National Institute of Health were used throughout these studies. The rabbits were housed in individual metal cages with half-inch wire mesh false bottoms. It

was necessary to teach the animals to eat the purified diets. This was accomplished by including 25-gm. rabbit pellets (the stock ration fed the breeding colony) with 50 gm. of the experimental diet for 2 days and by adding about 5 gm. of the diet to each 100 ml. of their water supply for 1 day at the beginning of the experiment. After this procedure, the rabbits with few exceptions ate the purified diets readily.

The components of the diets used in these studies are shown in table 1.

TABLE 1

Composition of purified diets.

	DIET NUMBER			
	672	673	674	675
	Grams per 100 gm. of diet			
Casein [1]	20	20	20	20
Wesson oil	5	5	5	5
Sucrose	55.4	55.4	55.4	55.4
Cellophane [2]	15.6	15.6	15.6	15.6
Salt mixture [3]	4	4	4	4
Supplement [4]				
	Milligrams per 100 gm. of diet			
Carotene [5]	87	87	261	261
Drisdol [6]	20	20	60	60
a-Tocopherol	7.5	7.5	22.5	22.5
Choline	200	200	600	600
Niacin (nicotinic acid)	20	..	60	..
Inositol	10	10	30	30
	Micrograms per 100 gm. of diet			
2-Methyl-4-naphthoquinone	75	75	225	225
Pyridoxin	700	700	2100	2100
Thiamine	700	700	2100	2100
Riboflavin	700	700	2100	2100
Calcium pantothenate	1500	1500	4500	4500

[1] Casein was leached with 3 changes of 2% acetic acid, dried over heat, ground and washed with 3 changes of 70% alcohol.

[2] 780 gm. of sucrose was dissolved in water to make a thick syrup then thoroughly mixed with 220 gm. of PT glycerine softened cellophane flakes (E.I. duPont de Nemours and Co.), dried over a low flame and finely ground in a burr mill.

[3] The salt mixture was prepared by the method described by Osborne and Mendel (J. Biol. Chem., vol. 37, p. 572 ('19)) except that the following changes were made: NaF was reduced to 1% of the original value and 0.2 gm. $CuSO_4$ was added.

[4] The water-soluble vitamins were dissolved in water and mixed thoroughly with the casein; the oil-soluble vitamins were then mixed with the Wesson oil and mixed with the casein water-soluble vitamin mixture. The sucrose-cellophane mixture was mixed with the salt mixture then mixed thoroughly with the casein-vitamin mixture.

[5] Carotene-in-oil 7500 U.S.P. units per gram.

[6] Drisdol contained D_3 40,000 units per ml. in propylene glycole.

Experiment 1. Six rabbits, approximately 10 weeks old, weighing from 1600 to 1850 gm. were placed on diet 672 and observed for 21 weeks. The average weight gain of these rabbits was rapid for 16 weeks when an average gain of 1173 gm. per rabbit was reached. Records kept by the breeders of the stock colony of rabbits used in these experiments show that the average gain in weight of the 10-week-old rabbits fed the stock ration is 1100 to 1300 gm. in 15 weeks; following this period the rate of weight gain is less. Little change in the average weight was noted during the remaining 5 weeks of observation (fig. 1). Considerable variation in the weight of individual rabbits was noted but this might be expected since the animals were not litter mates and were fed ad libitum.

Experiment 2. After it had been determined that rabbits would survive and grow well when fed this purified diet, studies were begun to determine if niacin was necessary for survival and growth. Eleven pairs of litter mates (nine pairs of males and two of females) were placed on study as they became available. The weights of the animals were from 1100 to 1850 gm. Litter mates of as near equal weight as practicable were used. The greatest difference was 230 gm. All of the rabbits were fed ad libitum. One animal of each litter received the niacin deficient diet 673 while its mate was fed the control diet 672. Assay of the diets for niacin by a slight modification of the method of Snell and Wright ('41) revealed that the control diet 672 contained 0.21 mg. of niacin per gram whereas the quantity in diet 673 was too low to be determined. These rabbits were observed for 19 weeks. Seven of the eleven rabbits fed the niacin deficient diet died before the end of the period of observation. When one of the animals died, its corresponding litter mate was withdrawn from the test. Two of the rabbits that were fed the niacin deficient diet gained 330 and 340 gm., respectively. Even with these gains the animals on the deficient diet maintained an average weight that was less than the starting weight. The litter mates that received the control diet grew at an almost normal rate (fig. 2).

The niacin deficient animals showed marked emaciation and the majority had diarrhea, frequently bloody, shortly before death. Careful examination of the mouth failed to reveal any significant changes in the buccal mucosa. At autopsy, the only consistent findings were liquefaction of the fecal matter and gaseous distension of the intestines. Occasionally ascites was present. It was felt that there was not enough evidence to regard these findings as pathognomonic of niacin deficiency.

Experiment 3. Six pairs of litter mates were placed on paired feeding. The diets employed were control diet 674 and niacin deficient diet 675.

These diets contained three times the amount of vitamins in diets 672 and 673 to assure protection against multiple vitamin deficiencies developing in animals fed restricted amounts of diet. After the first 2 or 3 weeks the animal on the niacin deficient diet ate the least and therefore controlled the amount of ration fed its litter mate. Each pair of rabbits was observed until one of the animals died at which time its litter mate was sacrificed. Three rabbits on the control diet 674 and three fed the niacin deficient ration 675 died before their respective litter mates. The length of time on the rations varied from 38 to 149 days.

The ration consumed varied between 29.1 and 42.6 gm. per day by the rabbits of the various pairs. The diet was calculated to contain 3.4 cal. per gm. Each rabbit, therefore, consumed from 99 to 144 cal. per day, or from 79 to 104 cal. per kilogram of body weight. Except for one pair of rabbits, nos. 139 and 140, the difference in weight change of the litter mates while on the test was comparatively small. Five of the rabbits receiving the niacin deficient diet showed an average loss of 110 gm. from the starting weight and their litter mates receiving the control diet lost an average of 150 gm. Rabbit no. 139 fed the deficient diet lost 560 gm. during the 149 days of the experiment while its litter mate on the control diet gained 590 gm.

The antemortem symptoms and postmortem findings among the rabbits dying in this experiment (diarrhea just before death and liquefaction of fecal matter and increased gas in the intestines at postmortem examination) were similar to those shown by the niacin deficient rabbits in the preceding experiment. These findings were shown by the rabbits that had received the control diet as well as those that received the niacin deficient diet. It is possible that the signs and symptoms shown by these rabbits, as well as those fed the niacin deficient ration in the previous experiment, were due to slow starvation brought about by anorexia when niacin was excluded from the diet.

Histologic examination of the tissues from rabbits in these two experiments were made.[1] The tissues examined were the heart, lungs, liver, pancreas, spleen, adrenals, kidneys, small and large intestines, hamstring muscles, sciatic nerve, lumbar, dorsal and cervical vertebrae (including muscle, bone, bone marrow and cord) and four levels of the brain.

Tissues were fixed in formalin, embedded in paraffin and stained with eosinated polychrome methylene blue and with van Gieson's con-

[1] The histological examinations were made by Surgeon (R) G. L. Fite and Passed Assistant Surgeon K. M. Endicott, Division of Pathology, National Institute of Health.

nective tissue stain. Weigert-Pal myelin stains of brain, spinal cord, and sciatic nerve were prepared. Marchi preparations or Sudan IV stains of frozen sections were made in a few cases.

There were no consistent lesions which could be attributed to niacin deficiency. Two of the thirteen rabbits examined receiving no niacin showed superficial degeneration of the mucosa of the colon consisting of cytoplasmic oxyphilia and nuclear karyorrhexis. Both rabbits (nos. 95 and 151) had diarrhea. Three other niacin deficient rabbits and one control rabbit had diarrhea but showed no lesions in the colon. Atrophy and necrosis of skeletal muscle, atrophy of fat, and hypocellularity of bone marrow were noted in several rabbits receiving niacin in the diet as well as in several of those receiving no niacin.

Assays for niacin were made on representative samples of liver, kidney, and muscles of the hind leg of nine of the eleven pairs of rabbits that were fed ad libitum and of the six pairs of animals that were pair fed. By referring to table 2 it will be noted that there was no significant difference of niacin content of the respective tissues of the litter mates that had been on tests less than 60 days. If all rabbits are considered, the difference in the niacin content of the muscles of the deficient and the control animals is of borderline significance. If, however, only the rabbits that were on diet more than 15 weeks are considered there is a definite diminution in the niacin content of the muscles of the deficient animals as compared to their control litter mates.

Experiment 4. The next study was to determine whether reversing the diets after the rabbits had been fed the niacin deficient or the control ration would effect their growth rate. One of each of four pairs of litter mates was fed the niacin deficient diet 673 and its mate the control diet 672. The diets were reversed at the end of 7 weeks and the rabbits that had received the niacin deficient diet were changed to the control diet, and those that had received the control diets were given the niacin deficient diets. At the time the groups were reversed the control rabbits had gained 560, 670, 880 and 330 gm., respectively, while those on the niacin deficient diet had a weight change of +10, −20, −160, and −260 gm. Following the reversal of the diets the rabbits that had been fed the control diet 672 but that now received the deficient diet usually gained less rapidly or showed a loss of weight. At the end of the next 7-week period these rabbits had a weight change of +120, +320, −60, and +10 gm., respectively, while those that had been fed the niacin deficient diet 673 but now received the control diet gained 690, 940, 790, and 800 gm. (fig. 3). Thus it is shown that if rabbits are fed a purified diet with adequate supplements of the more common

pure vitamins their growth is rapid whereas if the same diet is employed but without the addition of niacin as the only difference, the weight increase is small or loss of weight may occur.

Experiment 5. An experiment was next set up to determine the niacin requirement of rabbits when fed the purified diet.

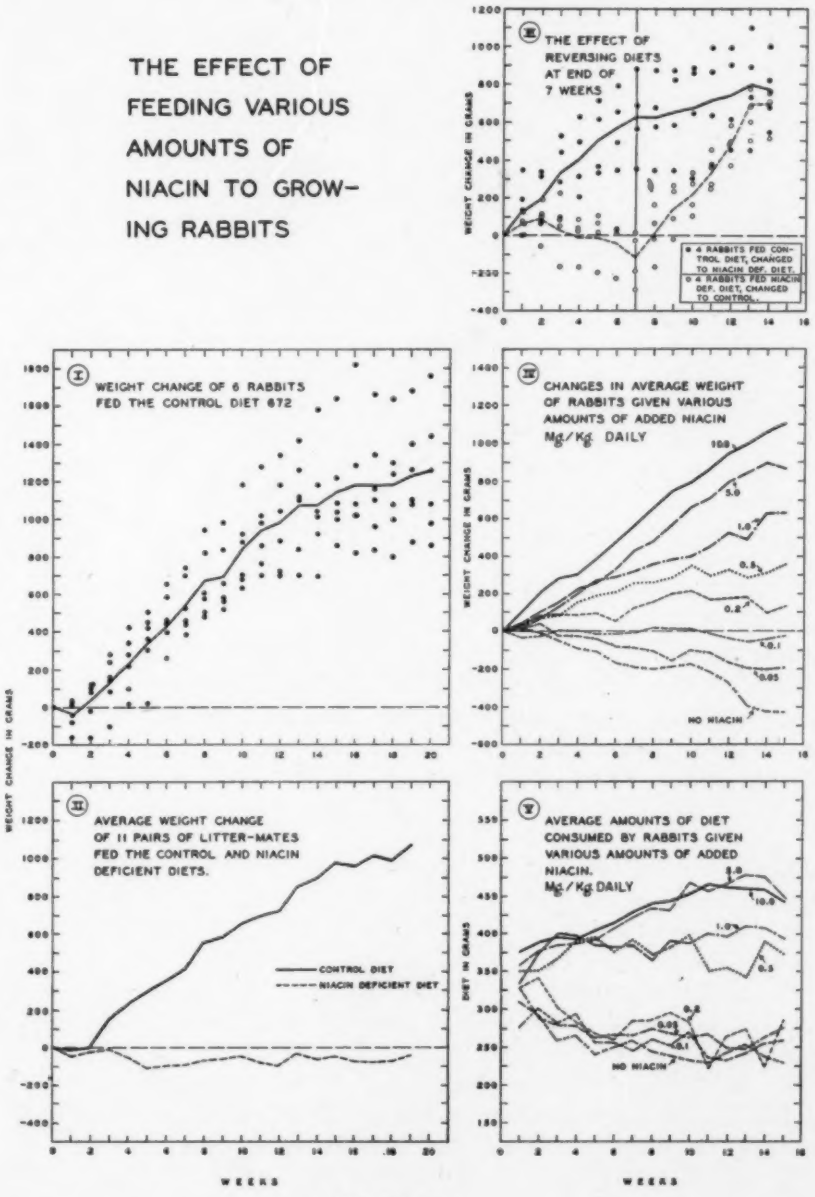
Ten groups of eight rabbits each, without regard to litter mates, were fed the niacin deficient diet 673. Supplements of niacin equal to 0.0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 mg. per kilogram of body weight daily were added to the diets of the rabbits in the respective

TABLE 2

Results of niacin assays of tissues of litter mate rabbits that had been fed niacin deficient and control diets.

PAIR NO.	NO. DAYS ON DIET	TISSUES ASSAYED	NIACIN CONTENT IN RABBITS FED		PAIR NO.	NO. DAYS ON DIET	TISSUES ASSAYED	NIACIN CONTENT IN RABBITS FED	
			Niacin-deficient diet	Control diet				Niacin-deficient diet	Control diet
Ad libitum feeding of niacin-deficient diet 673 and control diet 672									
			μg./gm. of tissue					μg./gm. of tissue	
89	180	Liver	187	150	151	44	Liver	120	175
and		Kidney	87	94	and		Kidney	78	100
90	180	Muscle	90	141	152	44	Muscle	115	130
93	259	Liver	135	242	153	47	Liver	150	140
and		Kidney	68	100	and		Kidney	105	110
94	259	Muscle	90	135	154	47	Muscle	105	125
95	195	Liver	175	190	155	156	Liver	125	170
and		Kidney	65	89	and		Kidney	110	112
96	195	Muscle	71	150	156	156	Muscle	104	135
99	107	Liver	80	160	157	51	Liver	100	120
and		Kidney	82	110	and		Kidney	100	110
100	107	Muscle	55	150	158	51	Muscle	120	120
149	175	Liver	198	248					
and		Kidney	112	83					
150	175	Muscle	67	135					
Paired feeding of niacin-deficient diet 675 and control diet 674									
137	94	Liver	140	144	143	38	Liver	120	110
and		Kidney	86	100	and		Kidney	95	80
138	94	Muscle	93	139	144	38	Muscle	100	...
139	147	Liver	150	167	145	119	Liver	166	166
and		Kidney	108	...	and		Kidney	73	144
140	147	Muscle	73	155	146	119	Muscle	80	...
141	110	Liver	165	195	147	40	Liver	110	140
and		Kidney	110	110	and		Kidney	75	110
142	110	Muscle	136	106	148	40	Muscle	120	110

THE EFFECT OF FEEDING VARIOUS AMOUNTS OF NIACIN TO GROWING RABBITS



Figures 1 to 5

groups three times each week. The animals were observed for 15 weeks. The weight of the rabbits and the ration consumed was recorded each week.

Considerable variations in the weight change of the individual rabbits of each group were observed. One of the eight rabbits that were supplemented with 1.0 mg. of niacin per kilogram of body weight daily, two of those that received 2.5 mg., three that were fed 5.0 mg., four that ate 7.5 mg., and seven of the eight animals that received the equivalent of 10.0 mg., grew at a rate comparable with the average rabbit that had received the control diet 672. Deaths occurred in each of the groups that were supplemented with 1.0 mg. of niacin/kg. of body weight or less. Each of these animals failed to grow and had diarrhea shortly before death. One rabbit in each group receiving 1.0, 2.5, and 5.0 mg. niacin, respectively, met with accidents and was discarded.

The average weight of the rabbits in each group increased with each added amount of niacin until the level of 10.0 mg./kg. of body weight daily was reached (fig. 4). This observation suggests that it may be possible to use the rabbit as a biological assay animal for niacin if significant numbers are employed.

The average weekly consumption of food by the groups that received 0, 0.05, 0.1 and 0.2 mg., respectively, of niacin per kilogram of body weight daily was less as the experiment progressed than at the beginning of the test. When 0.5 or 1.0 mg./kg. of niacin was given, the amount of food eaten was about the same throughout the period of observation. But when 5.0 or 10.0 mg./kg. was supplied, the average amount of diet consumed increased as the test progressed (fig. 5).

CONCLUSIONS

1. Young rabbits fed a purified ration supplemented with pure vitamin preparations grew at a nearly normal rate.

2. Rabbits fed this purified diet require niacin for survival and adequate growth.

3. Symptoms shown by rabbits receiving the niacin deficient diet are loss of weight and usually severe diarrhea 24 to 48 hours before death.

4. Rabbits fed the diet containing adequate amount of niacin but pair-fed with litter mates receiving the niacin deficient ration died as early as the deficient animals and showed simliar symptoms.

5. Rabbits fed the niacin deficient diet for 3 months or longer showed significantly less niacin in the voluntary muscles than the litter mates given niacin.

6. Under the conditions of our experiments rabbits fed the purified diet showed considerable individual variation in growth response to added niacin. Maximum average growth of groups of eight rabbits was not reached until an amount of niacin equal to 10 mg./kg. of body weight daily was added.

7. Groups of eight rabbits fed the niacin deficient diet supplemented with graduated amounts of niacin gave average growth responses directly related to the amount of niacin fed.

8. The only symptom which could be attributed to niacin deficiency was anorexia; the other symptoms manifested by the deficient rabbits may have been due to slow starvation.

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EFFECT OF SUNSHINE UPON THE ASCORBIC ACID AND RIBOFLAVIN CONTENT OF MILK [1]

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ONE FIGURE

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Milk is very generally considered a rich natural source of riboflavin for the human dietary. Milk also contains appreciable amounts of ascorbic acid. In an extensive review of the literature Booher, Hartzler and Hewston ('42) found that the ascorbic acid content of whole milk varied from 0.07 mg. to 29.2 mg. per 100 gm. and riboflavin varied from 60 μg. to 342 μg. per 100 gm., apparently depending upon the part of the world in which the milk was produced and upon the breed, feed and physical condition of the cow. However, data concerning the amount of ascorbic acid and riboflavin in milk when drawn from the cow do not supply the ultimate consumer with information regarding the amounts of these vitamins he obtains in the milk that he consumes. The influence of factors such as pasteurization, bottling, lapse of time, storage by the producer or dispenser, storage on the customer's doorstep and in the home refrigerator, operates between the time the milk is drawn from the cow and its ultimate use. Holmes and Holmes ('43) reported that milk of quite uniform riboflavin content could be produced by a sizeable mixed herd of cows maintained under standardized conditions, and Holmes ('44) found very little destruction of riboflavin when milk was pasteurized by the holding or flash process. On the other hand, Williams and Cheldelin ('42), Ziegler ('44), Peterson, Haig, and Shaw ('44) and Stamberg and Theophilus ('44) have submitted conclusive evidence that milk may lose a considerable portion of its riboflavin while it is exposed to sunshine on the consumer's doorstep. Unfortunately these investigators did not report information concerning the amount of the sun's energy that fell upon the milk bottles or the temperature of the milk during its exposure to sunshine. The study reported in this paper was conducted to accumulate data concerning these factors.

[1] Contribution no. 542 from the Massachusetts Agricultural Experiment Station.

The milk used in this study was supplied by the Dairy Manufacturing Laboratory where it was pasteurized at about 145°F. for 30 minutes. As soon as the milk was cooled it was bottled in one-half pint flint glass bottles and exposed to sunshine. The bottles, which varied from day to day, were assumed to be typical of those used by commercial dairymen. In order to provide uniform exposure conditions, the milk was placed on the flat roof of a four-story building 67 feet above the ground and thus all shadows were eliminated. The sample bottles of milk were arranged on a large sheet of brown manila paper so as to minimize any absorption or reflection of sunshine in the area surrounding the samples of milk.

The temperature of the milk when it was exposed to the action of sunlight varied from day to day but it was hoped that it was similar to that of commercial milk when delivered to the individual customer by the milkman. It was recognized that the effect of exposing milk to sunshine might be influenced somewhat by the temperature of the milk during exposure. Accordingly a record was made of the temperature of the milk and of the air surrounding the milk bottles at the beginning and end of the exposure periods. A thermometer was placed in the milk with the bulb at the intersection of the horizontal and vertical centers of the bottle. Inasmuch as the amount of sunshine which falls on milk bottles remaining on doorsteps varies with meteorological conditions, an attempt was made to expose milk on very cloudy days when there was little or no sunshine, on days of intense sunshine and on days when the sunshine was intermittent. There were two 30-minute or two 60-minute exposure periods for each bottle of milk and the second exposure followed immediately after the first.

The intensity of the sunshine to which the milk was exposed was measured continuously by a pyrheliometer equipped with an automatic recording device. The pyrheliometer was placed near the bottles of milk which were being exposed.

ASSAY METHODS

The effect of exposure of milk to sunshine was judged by the ascorbic acid and riboflavin content of the milk just previous to and immediately following exposure. Assays of the milk were commenced as soon as its exposure to sunshine was completed. The ascorbic acid content of the milk was determined by the method employed in previous studies, namely, that reported by Holmes, Tripp, Woelffer, and Satterfield ('39). The riboflavin assay procedure which was employed has been previously described by Holmes, Jones, Wertz, and Kuzmeski

('43) except that potassium permanganate and hydrogen peroxide solutions were used instead of stannous chloride and sodium hyposulphite to discharge any possible extraneous color in the milk.

RESULTS

Temperature. Detailed data concerning the conditions of the exposure of the milk to sunshine are reported in table 1. It will be noted that on different days the temperature of the air surrounding the milk at the start of the first exposure period varied from 18.0° to 30.0°C. At the

TABLE 1

Data concerning exposure of milk to sunshine.

SAMPLE	TEMPERATURE (DEGREES C)						INTENSITY OF SUNSHINE (GM. CAL. PER SQ. CM. PER MINUTE)					
	First period				Second period		First period			Second period		
	Start		End [1]		End		Minimum	Maximum	Average	Minimum	Maximum	Average
	Air	Milk	Air	Milk	Air	Milk						
1	18.0	9.5	18.0	15.0	20.0	18.0	.00	.13	.08	.13	.23	.14
2	20.0	9.0	22.0	18.5	24.0	22.0	.13	.36	.29	.35	.46	.39
3	18.5	11.5	21.0	19.0	20.0	23.0	.20	.58	.33	.58	1.14	.90
4	26.5	9.0	28.5	19.0	31.0	26.5	.59	.76	.67	.59	.87	.76
5	24.5	8.0	25.5	18.0	27.0	24.0	.43	.94	.73	.86	1.09	.98
6	23.5	9.5	26.0	22.5	29.0	27.5	.35	.42	.37	.40	.64	.46
7	22.5	8.0	25.0	18.0	26.0	25.0	.81	.99	.93	.84	1.06	.88
8	24.0	10.0	25.5	21.0	25.0	24.5	.94	1.09	.99	1.07	1.22	1.13
9	27.0	11.5	27.0	26.0	33.0	33.5	.28	.86	.56	.50	1.19	.93
10	20.5	10.5	22.0	20.0	26.5	27.5	.26	.99	.58	.45	1.52	1.28
11	30.0	10.5	33.0	30.5	34.5	38.5	.76	.96	.88	.96	1.09	1.06
12	23.5	7.0	28.0	23.5	28.5	31.0	.85	1.22	1.00	.99	1.34	1.24
13	27.5	9.0	28.5	27.5	30.0	32.0	.99	1.19	1.13	1.19	1.39	1.28

[1] Readings for end of 1st period and start of 2nd period identical.

Samples 4, 5, 7 and 8 were exposed for two 30-minuute periods.

All the other samples were exposed for two 60-minute periods.

end of the first period the temperature varied from 18.0° to 33.0°C., and at the end of the second exposure period, which immediately followed, the temperature range was from 20.0° to 34.5°C. The temperature of the 13 samples of milk when first exposed to sunshine varied from 8.0° to 11.5°. At the end of the first period the temperature ranged from 15.0° to 30.5° and by the end of the second exposure period the temperature of the milk varied from 18.0° to 38.5°C. It is obvious from these data that the different samples of milk were exposed to sunshine

under a wide variety of conditions. In general the temperature of the milk increased during the first hour to within 3° or 4° of the temperature of the air surrounding it. By the end of the second hour the temperature had increased to about that of the surrounding air and in some instances exceeded it depending upon the intensity of the sunshine, the wind velocity and temporary cloud effects. Also, probably the milk bottles tended to develop the greenhouse effect, namely, as in the case of greenhouses the amount of heat from the sun that entered the bottles exceeded that which left them.

Intensity of sunshine. In order that the variability of the intensity of the sunshine to which the milk was exposed may be visualized, data are recorded in table 1 concerning the minimum, maximum and average gram calories per square centimeter per minute for each sample of milk during the first and second exposure periods. In an attempt to obtain as much variation of sunshine intensity as possible some samples (4, 5, 7 and 8) were exposed to sunshine for two 30-minute periods, and the remaining samples were exposed for two 60-minute periods. The total amount of sunshine that fell upon the bottles of milk was computed by multiplying the average gm. cal. per sq. cm. for the period by the number of minutes of exposure. The sunshine varied during the exposure of the different samples of milk from a total of 4.8 gm. cal. per sq. cm. for the initial period for sample 1 to 144.6 gm. cal. per sq. cm. for the combined periods for sample 13. It was relatively easy to obtain a total of less than 70 gm. cal. per sq. cm. during a 1- or 2-hour period, but it was not convenient to obtain many exposures of milk at higher sun intensities during the experimental period and it did not seem feasible to extend the period since under practical conditions probably milk is not allowed to stand on the customer's doorstep for more than 2 hours of sunshine.

Effect of sunshine on the reduced ascorbic acid in milk. The ascorbic acid content of the samples of milk at the beginning of the exposure period varied from 13.6 to 16.8 mg. per liter. The value 5.2 mg. per liter for sample 11 should not be considered normal because the sample stood for about 4 hours exposed to light in the laboratory on a very warm day before it was assayed. The amount of ascorbic acid in the milk at the end of the first period varied from 0.0 to 4.8 mg. per liter. At the end of the second exposure period only two samples contained any reduced ascorbic acid. It is apparent from these data that reduced ascorbic acid in milk is very rapidly oxidized when the milk is exposed to sunshine. In fact, samples 4, 5, 7 and 8 showed no ascorbic acid after 30 minutes exposure to bright sunshine.

This observation is in agreement with those of Kon and Watson ('36) who found that milk exposed to June skyshine lost all its reduced ascorbic acid within the first hour of exposure. They concluded that in general a pint bottle of milk exposed under practical conditions for one-half hour in the sun loses fully one-half of its original antiscorbutic properties. The rapid loss of ascorbic acid noted in this study when milk was exposed to sunshine is in accord with earlier observations by Krauss ('40) who reported that pasteurized milk kept in a clear bottle at room temperature lost practically all of its vitamin C within 6 hours. Diemair and Fresenius ('40) were much impressed by the possible rapid destruction of vitamin C, for they reported the loss of vitamin C by exposure of milk to light while carrying out analyses Concerning the cause of the loss of reduced ascorbic acid from milk exposed to light or sunshine Buruiana ('37) stated that the action of sunlight on milk produces oxidation of unsaturated fat, and oxidation by catalytic dehydrogenation of the ascorbic acid present in the milk; and Hand, Guthrie and Sharp ('38) have stated that lactoflavin is the sole agent in milk responsible for the sensitivity of ascorbic acid to light. In a study of the pigments, vitamins and enzymes of milk in relation to changes in flavor and nutritive value, Hand and Sharp ('41) found that "riboflavin, the fluorescent green coloring matter in whey, is responsible for the oxidation of vitamin C in light."

However, these data do not prove that such milk is devoid of biological vitamin C value. Washburn and Krauss ('38) reported that milk stored 6 hours and 24 hours in ordinary milk bottles in a cooler with uncertain refrigeration showed, respectively, a 12.9% and 29.4% loss of ascorbic acid, measured by titration; but in a corresponding biological test with guinea pigs, in which light and temperature were not controlled during the storage of the milk, they did not find a corresponding decrease in the biological activity of the stored milk. Hand ('43) reported that light causes an oxidation of the reduced ascorbic acid in milk to dehydroascorbic acid which has biological value but the amount present in milk at any time depends on the rate of formation and the rate of destruction of dehydroascorbic acid. Kon and Watson ('36) found that exposure of milk to light for ½ hour destroyed all the reduced ascorbic acid and in the absence of light the larger portion of the dehydroascorbic acid disappeared within the succeeding 5 hours.

It is quite evident from the cited data and those assembled in this study that milk exposed to sunshine on the consumers' doorstep may lose much of its value as a source of vitamin C.

Effect of sunshine on the riboflavin in milk. It was anticipated that sunshine would have a significant destructive effect on the riboflavin content of milk, for Hogan and Hunter ('28) found that riboflavin was destroyed by exposure to ultra-violet rays of a quartz mercury vapor lamp for 10 hours and György ('35) reported that lactoflavin was destroyed by visible light. However, Fuhr, Dornbush and Peterson ('43) reported that experimental and market milk irradiated with ultra-violet light to produce 400 U.S.P. units of vitamin D per quart were not reduced in riboflavin content.

TABLE 2

Destructive effect of sunshine on the ascorbic acid and riboflavin content of milk. (mg./liter)

SAMPLE	ASCORBIC ACID			RIBOFLAVIN		
	Start	End 1st period	End 2nd period	Start	End 1st period	End 2nd period
1	16.2	4.8	0.8	1.88	1.69	1.41
2	15.7	0.4	0.0	1.53	1.11	.67
3	14.0	0.0	0.0	1.74	1.44	.66
4	14.9	0.2	0.0	1.89	1.52	.86
5	16.0	0.0	0.0	1.66	1.22	.86
6	16.3	0.8	0.0	1.70	1.28	.58
7	15.5	trace	0.0	1.69	1.18	.62
8	13.6	0.0	0.0	1.90	1.28	.69
9	16.4	0.4	0.0	1.72	.99	.41
10	14.4	..	0.4	1.57	1.14	.43
11	5.2[1]	0.0	0.0	1.57	.24	.25
12	16.8	0.0	0.0	1.48	.60	.30
13	15.9	0.0	0.0	1.78	.64	.36

[1] Sample 11 stood several hours in a warm room before assay.

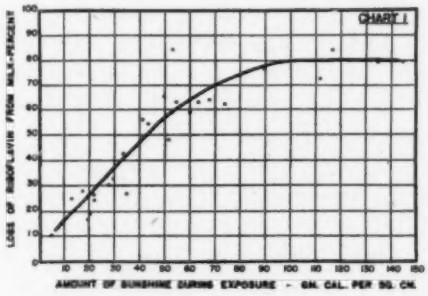
Samples 4, 5, 7 and 8 were exposed for two 30-minute periods.

All other samples were exposed for two 60-minute periods.

The amount of riboflavin (table 2) in the milk at the beginning of exposure to sunshine varied from 1.48 mg. to 1.90 mg. per liter. At the end of the first period of exposure it varied from 0.24 mg. to 1.69 mg. and at the end of the second period of exposure the riboflavin content of the milk varied from 0.25 mg. to 1.41 mg. per liter. The smallest destruction of riboflavin, 10.1%, occurred during the first period of exposure of sample 1 on a rainy day, when the temperature of the milk varied from 9.5° to 18.0°C. The largest destruction, 84.5% for sample 11, occurred on a very warm day when the temperature of the milk was 38.5°C. at the end of the second exposure period. The data accumulated in this study indicate that the temperature of the milk influences the amount of

destruction of riboflavin. This observation is in accord with those of Williams and Cheldelin ('42) who reported that increase in temperature accelerated the destruction of riboflavin, that the destruction of riboflavin in liquid foods proceeds at a rapid rate and that as much as 26% of the riboflavin in milk was destroyed by light in a 5–minute exposure at 100°C. Ziegler ('44) also found that the destruction of riboflavin in milk increases with temperature.

In order to facilitate comparing the effect of increasing the sun's intensity with the loss of riboflavin, the data concerning these factors are reported in chart 1. The points on the chart represent data for the intensity of the sunshine, the temperature of the milk, and the loss of riboflavin during the various exposure periods. The curve was drawn by inspection to represent the general average of the relation between the intensity of the sunshine and the riboflavin loss irrespective of the



temperature of the milk. Inspection of the chart will reveal that in general as the intensity of the sunshine to which the milk was exposed increased, the riboflavin destruction increased quite consistently until 60–70% of the riboflavin was destroyed when the milk was exposed to 50–70 gm. cal. per sq. cm. There was a further, though very slow, increase in the destruction of riboflavin as the intensity of the sunshine increased from 70–140 gm. cal. per sq. cm. It is interesting to compare these findings with those of other investigators. Peterson, Haig and Shaw ('44) obtained as much as 48% destruction of riboflavin when fresh milk was exposed in pint bottles from mid-morning to mid-afternoon to direct sunlight on an open porch at temperatures of 60°F. to 72°F. Ziegler ('44) exposed several types of milk, 20 hours after bottling in quart bottles, to direct mid-morning spring sunshine in the open air at atmospheric temperatures between 16.7° to 20.6°. His pasteurized milk lost 54% of its riboflavin during 2 hours exposure. Stamberg and

Theophilus ('44) reported as much as 80% loss of riboflavin from milk exposed in clear glass quart bottles to March sunlight, 45°F., for 6 hours. When exposed for 2 hours the loss was 48.3%. However, such a comparison should include allowance for the difference in the surface area of the milk in half-pint, pint and quart bottles.

SUMMARY

A study has been made of the effect upon the ascorbic acid and riboflavin of exposing milk in commercial one-half pint bottles to the action of sunshine. The intensity of the sunshine was measured with a pyrheliometer equipped with an automatic recording device. The milk was exposed for two 30- or two 60-minute periods. The "sunshine" varied from a total of 4.8 gm. cal. per sq. cm. on a rainy day to 144.6 gm. cal. per sq. cm. on a bright day. The temperature of the milk varied from day to day depending upon velocity of the wind, greenhouse effect of the milk bottles and intensity of the sunshine.

The destruction of reduced ascorbic acid was very rapid, for little, if any, was present after 30 minutes exposure. The riboflavin disappeared more slowly than the ascorbic acid. There was a 10% loss during 60 minutes exposure on a rainy day and about 85% loss during exposure to bright sunshine for 120 minutes. In general the destruction of riboflavin increased fairly consistently with the increase in intensity of the sunshine until 60–70% of the riboflavin was destroyed when the milk was exposed to a total of 50–70 gm. cal. per sq. cm. Increasing the sunshine from a total of 70 to 140 gm. cal. per sq. cm. caused a slow increase in the destruction of riboflavin.

These data show that milk allowed to stand for more than a short period on the consumer's doorstep exposed to strong light or sunshine is likely to lose a large amount of its ascorbic acid and riboflavin.

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CITRATE METABOLISM OF PRESCHOOL CHILDREN

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The data presented here are part of a larger study on the influence of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of preschool children. This report presents data on the effect of supplements of ascorbic acid, potassium citrate, and orange juice on the excretion of citric acid by four children studied during 1941–42. The effect of these supplements on ascorbic acid excretion has been reported by Meyer and Hathaway ('44), and their effect on calcium, phosphorus, and nitrogen metabolism will be reported later.

EXPERIMENTAL

Plan of the experiment

The general plan of the complete experiment, including a description of all subjects and of the diets used, has been reported in detail by Meyer ('43) and summarized by Meyer and Hathaway ('44). The subjects for the citrate study included a boy, E, and three girls, F, G, and H. (Their respective ages and weights at the beginning of the study were 55, 49, 40 and 38 months, and 34, 40, 32, and 29 pounds.) They lived in the college laboratory-apartment for 5 months and were maintained on a basal diet adequate in all nutrients except ascorbic acid. Citric acid determinations were made for sixteen 7-day periods (periods 5 through 20). Milk and all supplements were given at the same level to all subjects, but somewhat less of the basal foods was consumed by H throughout the study, and by G during the last 5 weeks. Ry-Krisp or Toasted Wheat Wafers were allowed ad libitum. Supplements of crystalline ascorbic acid, potassium citrate, and orange juice were given as indicated in table 1. The 3.38 gm. of potassium citrate corresponds roughly to the amount of citric acid and its salts calculated to be present in 200 ml. of orange juice and to be associated with 100 mg. of ascorbic acid from this source. The amount of orange juice actually given was determined daily as that amount which would furnish 110 mg. of ascorbic acid. Ten mg. of the ascorbic acid of the orange

juice replaced an equal amount of crystalline ascorbic acid included in the basal diet in all other experimental periods; the accompanying increase in citric acid and its salts was slight.

TABLE 1

Average daily intake and urinary excretion of citrates in four preschool children.

SUBJECT		E		F		G		H	
Diet	Period	Intake	Urinary Excretion	Intake	Urinary Excretion	Intake	Urinary Excretion	Intake	Urinary Excretion
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	7	1390	55	1390	260	1390	66	1312	81
	8	1337	56	1334	236	1334	105	1256	79
	9	1435	53	1432	244	1432	130	1339	119
	Av.	1387	55	1385	247	1385	100	1302	93
II	5	1385	63	1387	231	1387	109	1294	87
	6	1329	76	1329	230	1331	70	1239	59
	10	1415	66	1412	348	1411	74	1330	104
	11	1451	95	1448	250	1444	94	1367	77
	12	1535	62	1532	261	1528	84	1445	123
	Av.	1423	72	1422	264	1420	86	1335	90
III	13	3496	199	3493	341	3485	221	3399	210
	14	3511	225	3509	379	3498	237	3431	300
	15	3570	235	3567	416	3553	272	3486	268
	Av.	3526	220	3523	379	3512	243	3439	259
IV	16	3600	236	3598	423	3495	275	3504	263
	17	3568	236	3568	472	3464	292	3474	239
	18	3690	283	3687	514	3577	278	3586	186
	Av.	3619	252	3618	470	3512	282	3521	229
V	16	3600	236	3598	423	3495	275	3504	263
	20	3599	140	3596	357	3498	236	3498	151
	Av.	3700	157	3697	390	3598	238	3600	153

I. Basal diet (containing 10 mg. crystalline ascorbic acid).
II. Basal diet plus 100 mg. ascorbic acid.
III. Basal diet plus 3.38 gm. potassium citrate (citrate content equivalent to 2.19 gm. citric acid).
IV. Basal diet plus 100 mg. ascorbic acid plus 3.38 gm. potassium citrate.
V. Basal diet without crystalline ascorbic acid, but plus orange juice containing 110 mg. ascorbic acid and an average citrate content equivalent to 2.29 gm. citric acid.

Collection and preparation of samples for analyses

Urines. Half of each urine specimen was preserved with thymol for pH determinations and for citric acid and mineral analyses. At the

end of 24 hours the pH of the sample for that period was determined; then sufficient hydrochloric acid was added to make a 2% solution, and two-fifths of the sample was saved for the weekly composite specimen later used for citric acid and mineral analyses. All samples were stored under refrigeration.

Foods. At each meal aliquots were taken of all basal foods except milk. These were placed in Pyrex flameware saucepans and digested with 1:4 hydrochloric acid according to the method of Stearns ('28–'29). Composite samples for a weekly period were brought to volume in three 2000 ml. volumetric flasks, and aliquots were stored for analysis. The proteins of the food digests were removed by precipitation with trichloracetic acid at the time of analysis. Samples of Ry-Krisp and Toasted Wheat Wafers were partially digested with hydrochloric acid and then treated in the same manner as other food digests. Samples of the milk for each week [1] were also analyzed separately. The milk sera were prepared for analysis by the method of Lampitt and Rooke ('36).

Citric acid determinations

The Kuyper and Mattill ('33) modification of the pentabromacetone method was used for the citric acid determinations.

RESULTS AND DISCUSSION

Urinary excretion of citrates

The average daily values for the intake and urinary excretion of citrates (calculated as citric acid) by the four subjects are given by periods and by supplements in table 1. The intakes for all subjects were similar, but the excretion values for subject F were markedly higher than those for the other three children, regardless of supplement. The values for G and H were in the same range for diets I, II, and III, but those for H were lower on diets IV and V. The values for E were lower than those for G and H on diets I, II, and III, and between the G and H values on diets IV and V.

A comparison of the intakes and urinary excretions of citrates per kilogram of body weight was made. The average intakes per kilogram at the basal citrate level varied from 75 mg. (for F) to 96 mg. (for H); when potassium citrate was added to the basal diet, the intakes varied from 184 mg. to 244 mg. (for F and H, respectively). The basal citrate excretion values ranged from 3 to 9 mg./kg. for E, G, and H, but from

[1] Specially prepared by Prof. E. S. Guthrie so that it retained its ascorbic acid content and fresh flavor for at least a week. See Sharp, Hand and Guthrie ('39).

13 to 19 mg. for F. When potassium citrate was added to the basal diet, the citrate excretions for E and G ranged from 12 to 18 mg./kg., for H from 13 to 22 mg., and for F from 18 to 26 mg. When orange juice was added to the basal diet the excretion values for all the children were lower than on the potassium citrate supplement, averaging 9 to 14 mg./kg. for E, G, and H, and 19 mg. for F. The excretion values for F were, with one exception (H on diet III), still markedly higher than those for the other children. It is interesting to note that on a per kilogram basis, subject F, who generally maintained the highest excretion values, consistently had the lowest citrate intake. This is in agreement with the observations of Östberg ('31), substantiated by those of other workers, that the citrate excreted is of endogenous origin, and is independent of the citrate intake.

The individual variations among our subjects on a standardized diet are not surprising. Meyer and Smith ('40) in a study on rats noted considerable variation in citric acid production within a group on a given diet. They also observed that the fluctuations for any one rat were within narrow limits, so that a distinct individuality was discernible.

The values for the urinary excretion of citrates observed with these children correspond to those cited in the literature. McClure and Sauer ('22) observed excretions of 64 to 254 mg. for children of this age range (3 to 5 years) on uncontrolled citrate intakes. Sherman, Mendel, and Smith ('36) reported excretion values for normal adults of 356 to 1180 mg., or 5 to 20 mg./kg. In our subjects the values per kilogram varied from 3 to 26 mg., but only 2 values (3% of the total) were more than 2 mg. outside the limits given for adults.

The effect of changes in the ascorbic acid content of the diet on the citrate excretion

Interest in the possible interrelationships of ascorbic acid and citric acid was aroused by the papers of Purinton and Schuck ('43a, '43b). From the data in table 1 the possible effect on citrate excretion of adding 100 mg. of ascorbic acid to the basal diet or to the basal diet supplemented with potassium citrate is observable. The average excretion values were higher in five and lower in three of the eight cases. Statistical treatment of the data, however, indicates that the differences noted were significant only for F on the higher level of citrate intake (odds greater than 30:1, using Student's "t" table). It is realized that statistical treatment has limited value when so few cases are compared, but it does confirm the conclusions drawn by inspection of the

data, that the addition of ascorbic acid did not cause consistent increases in citrate excretion.

Comparison of the ascorbic acid "utilization"[2] values (Meyer and Hathaway, '44) with the citrate excretion values reveals that subject E showed the greatest increase in "utilization" of ascorbic acid at both levels of citrate intake, and showed no tendency to lowered citrate excretion. Subject H, who showed the least increase in "utilization" of ascorbic acid, showed no tendency to increased citrate excretion. Thus the results of this study do not appear to be in agreement with those of Purinton and Schuck ('43b) on human subjects. However, since our citrate values are averages rather than daily values, possibly there was an adjustment to the changed intake with time.

The effect of addition of potassium citrate to the diet on citrate excretion

The various supplements to the basal diet make possible a number of comparisons of the citrate excretion at different levels of citrate intake, e.g., the differences in excretion using potassium citrate as the source of added citrate (1) on a low ascorbic acid intake, (2) on a high ascorbic acid intake, and (3) ignoring the ascorbic acid intake.

An increase in citric acid excretion on the administration of potassium citrate was noted for all subjects; and, if the effect of the added citrate is considered rather than that of the total citrate, the increment of citric acid excreted was similar for all subjects. At the basal level of ascorbic acid intake, the addition of potassium citrate resulted in an increase in citrate excretion of 132 to 166 mg., (average excretion on diet I subtracted from that on diet III) or 6.2 to 7.8% of the increased intake. At the higher level of ascorbic acid intake this difference amounted to 139 to 206 mg., (average citrate excretion on diet II substracted from that on diet IV) or 6.4% to 9.4% of the added citrate. When the changes in ascorbic acid are ignored, and the changes in citrate level only are considered (average of all values for citrate excretion on diets I and II substracted from the average on diets III and IV) the range of values is even smaller, 153 to 167 mg., or 7.1 to 8.1%.

Schuck's studies ('34b) on the effect of sodium citrate on the citrate excretion in four adults may be compared with the present study on the affect of added potassium citrate. Sodium citrate equivalent to 12 gm. of citric acid was added to her basal diet. Of this, 856 to 1898 mg. were excreted, equivalent to 7.1 to 15.8% of the added intake. Subject S

[2] The term "utilization" is used arbitrarily to refer to the difference between intake and excretion: corresponds to the term "retention" in the reports of Purinton and Schuck.

showed exceptionally high excretion except on the basal diet. If her values are omitted, the range of excretion with the sodium citrate was 7.1 to 9.1%, or is similar to the range found in the present study with potassium citrate.

The effect of addition of citrus fruits to the diet on citrate excretion

At the higher level of ascorbic acid intake the addition of citrate as orange juice resulted in an increase in citrate excretion ranging from 63 to 126 mg. (average citrate excretion on diet II substracted from that on diet V) or 2.8 to 7.0% of the added intake. For each subject the increase in excretion was less when the citrate was added as orange juice than when it was added as potassium citrate.

In 6 subjects given 1000 ml. of orange juice daily, Schuck ('34a) found an excretion of 234 to 1294 mg., or 2.6 to 16.6% of the added orange juice. Again, if the values for subject S are omitted, the range was 234 to 443 mg., or 2.6 to 7.5%. Lanford ('42) reported that with the addition of 1800 ml. of grapefruit juice the range of "extra" citrate found in the urine of her subjects was 112 to 738 mg., or 0.67 to 3.42% of the increased intake. The range found in the present study when orange juice was added to the basal diet (2.8 to 7.0%) is similar to that found by Schuck ('34a).

Relationship of citrate excretion to acid-base balance

Changes in the ascorbic acid content of the diet made little or no change in the pH of the urine, and also caused little change in the citrate excretion in the four subjects of this study. The addition of potassium citrate to the diet, however, increased the average urinary pH by 0.9 to 1.4 units, from averages of 5.5 to 5.9, to from 6.8 to 6.9. The change in urinary citrate excretion corresponding to these pH changes was an increase of 153 to 171 mg. The addition of orange juice increased the basal pH values by 0.3 to 0.6 units, to averages of 6.0 to 6.2, and the urinary citrate excretions by 63 to 152 mg.

Probably the suggestion that urinary excretion of citrates is related to the pH of the urine was first made by Östberg ('31). He observed that ingestion of either sodium citrate or sodium bicarbonate increased the citrate excretion, and concluded that the more alkaline urine was responsible for the increase in both cases. His experiments have been extended and his conclusions confirmed by others.

In Lanford's study ('42) using grapefruit juice as the source of citric acid, she found for the 10 subjects on controlled intake, an increase in the pH of the urine of 0.53 ± 0.11 units over the average of 6.00 on the control days. She gives statistical evidence that the increased citrate excretion and the rise in urinary pH were interdependent.

Schroeder and Smith ('43), in a study with rats, showed that on two isocaloric diets, containing 6 and 27 mg. of citric acid per period, the addition of graded amounts of sodium bicarbonate resulted in the excretion of similarly graded amounts of citric acid, independent of the citric acid intake. They have suggested that there is a direct relationship between the amount of alkali administered to the rat, and the excretion of endogenous citric acid, and that the total urinary citric acid is dependent on the degree of alkalosis.

In the present study the increase in the intake of citrates was, for each child, about 80 mg. more when orange juice was given than on the potassium citrate supplement, but the excretion was decreased by 44 to 95 mg. It has been pointed out that the pH of the urine was less alkaline with the orange juice than with the potassium citrate supplement. These observations confirm the conclusion that the acid-base relationship is more important than the citrate content of the diet in controlling citrate excretion.

SUMMARY

The citric acid metabolism of four preschool children was studied over an experimental period of 16 weeks. The children were on a controlled diet which was supplemented with 100 mg. of ascorbic acid, 3.38 gm. potassium citrate, or equivalent amounts of orange juice as indicated. The results were as follows:

1. On the basal diet the four children excreted an average of 55, 93, 100 and 247 mg. citric acid in the urine, or 3, 6, 7, and 13 mg./kg. of body weight.

2. The addition of 100 mg. of ascorbic acid to the basal diet did not cause consistent changes in citrate excretion, and no relationship was found between ascorbic acid "utilization" and citrate excretion.

3. The addition of 3.38 gm. of potassium citrate to the basal diet, with or without the addition of ascorbic acid, caused a definite increase in excretion of citrates, consistent in amount for all four children, averaging 153 to 167 mg., or 7.1 to 8.1% of the added citrate intake.

4. The addition of an amount of orange juice containing an amount of citrate approximately equivalent to that in the potassium citrate supplement caused an average increase in citrate excretion of 63 to 126 mg. or 2.8 to 7.0% of the added citrate intake.

5. The increase in citrate excretion on the addition of potassium citrate or orange juice to the diet appears to be related to the rise in alkalinity of the urine rather than to the citrate content of the diet.

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FURTHER STUDIES ON CYSTINE, METHIONINE AND CHOLINE IN CHICK DIETS

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The dietary requirements of the chick for the sulfur-bearing amino acids are met by levels of 1.0 to 1.1% of cystine plus methionine, if half of this total is supplied by methionine, and the diet contains adequate amounts of choline (Grau and Almquist, '43). Under certain conditions, methionine and betaine (glycine betaine) can replace a portion of the choline required in the diet (Almquist and Grau, '44), but replacement of methionine by dietary excesses of choline and cystine has not been thoroughly investigated.

Results of further studies on the interrelationships of the sulfur-containing amino acids may now be reported. The chicks used were not depleted of choline as before, but were fed a practical rearing mash from the time of hatching until they were segregated into groups of 5 and given the experimental diets; otherwise, the methods and diets were the same as those previously detailed (Grau and Almquist, '43).[1] A summary of the results is given in table 1.

At the low level of 1.7 millimols of added sulfur amino acids with choline present, methionine or cystine elevated the growth rate from a basal value of approximately 1% to a suboptimal plateau value of approximately 4% (fig. 1, Grau and Almquist, '43). The apparent equivalence of cystine and methionine in these cases was due to an extreme deficiency of cystine and a suboptimal level of methionine in the basal diet. The deficiency could be relieved by additional methionine, which is a precursor of cystine, or by cystine itself. When 2.5 millimols were added as cystine only (table 1, diets 15 and 44), the growth rate remained near the 4% value even with a high level of choline. When 3.3

[1] To facilitate understanding the significance of the data now being presented, a summary of the basal diet follows: isolated soybean protein 23, glucose 52.8, cellulose (Cellu Flour) 5, calcium gluconate 8, mineral mixture 4.24, cottonseed oil (Wesson) 5, cod liver oil 1, and crystalline sources of vitamin K, thiamine, riboflavin, pyridoxine, nicotinic acid and calcium pantothenate. Biotin was provided by a concentrate. The soybean protein provided 20 gm. of protein per 100 gm. of diet; the methionine and cystine contents of the basal diet were 0.3% and 0.05%, respectively.

millimols of cystine were added (diets 45 and 46), gains were not appreciably changed from the 4% value at any level of added choline chloride up to 0.6%. When half of this cystine was replaced by methionine (diet 20) the gain per day was distinctly higher. At the 4.2 millimol level of added cystine (diet 47) the gain per day was not appreciably raised over 4%, and here, again, an excess of choline did not allow better growth.

TABLE 1

Effect of various added levels of cystine, methionine and choline on the rate of gain of chicks fed a basal diet deficient in all three compounds.

DIET NUMBER	SUPPLEMENTS ADDED TO THE BASAL DIET [1]			TOTAL MILLIMOLS SULFUR AMINO ACIDS ADDED PER 100 GM. DIET	NUMBER OF GROUPS	AVERAGE PER CENT GAIN PER DAY
	l (−)-Cystine	dl-Methionine	Choline chloride			
14 [2]	0.20	...	0.20	1.7	2	4 0
15 [2]	0.30	...	0.30	2.5	1	4.0
44	0.30	...	0.60	2.5	1	4.1
45	0.40	...	0.20	3.3	1	3.6
46	0.40	...	0.60	3.3	1	3.9
20 [2]	0.20	0.25	0.20	3.3	1	5.2
47	0.50	...	0.60	4.2	1	4.1
48	0.60	...	...	5.0	1	0.7
49	0.60	...	0.05	5.0	2	3.8
50	0.60	...	0.20	5.0	3	4.1
51	0.60	...	0.40	5.0	2	4.3
52	0.60	...	0.60	5.0	2	4.2
53	0.50	0.12	0.20	5.0	2	5.3
22 [2]	0.20	0.50	0.20	5.0	1	6.0
54	0.20	0.50	0.60	5.0	1	5.8

[1] Expressed in % added to diet.

[2] From Grau and Almquist ('43). The diet numbers were assigned previously. Chicks in these groups had been depleted of choline to a perotic stage.

When 5 millimols of cystine were added (diets 49, 50, 51 and 52), gains were essentially the same as before. The levels of choline chloride in these diets varied from 0.05 to 0.60%. The very poor growth with diet 48, to which no choline had been added, indicates that an extreme choline deficiency existed in the basal diet. When as little as 0.1% cystine was replaced by an equivalent amount of methionine, gain was increased to 5.3% (diet 53), while increasing the methionine still further (diets

22 and 54), resulted in gains of approximately 6%, the best rate obtained with this type of diet.

The data of table 1 show that gradual increases of cystine up to 3 times 1.7 millimols of added amino acids, and simultaneous increases of choline up to 12 times the minimum amounts which would support the plateau value of growth rate did not appreciably increase this growth rate above 4%.

These results, together with our former reports, present a fairly clear basis of understanding the interrelations of cystine, choline and methionine in the chick. During acute deficiencies of cystine and choline the chick effects the best adjustment possible in utilizing methionine for all three requirements. Whenever methionine must be partially utilized to fulfill the functions of cystine and choline, additions of cystine and choline may promote additional growth through sparing methionine. When this sparing action has been completely fulfilled at any suboptimal level of methionine, further additions of cystine or of choline are of no avail. Growth then cannot be accelerated except by additions of methionine. It is probable, however, that for each increment of methionine, small increases of cystine and of choline will show favorable effects on growth provided the amounts already present are balanced with the level of methionine.

Much has been implied concerning the possibility of using choline to improve diets in cases of inadequate methionine content. It is now evident that favorable effect from such supplementation could only be expected under circumstances where methionine is necessarily being used to provide some of the functions of choline in the diet, or in other words, where there is also a labile methyl deficiency. Our data show distinctly that an inadequate level of methionine cannot be compensated for by excessive levels of cystine and choline, and there is no evidence for a methionine synthesis (or interference with metabolic methionine destruction) through the presence of surpluses of these compounds in the diet.

SUMMARY

Through the use of a diet deficient in methionine and cystine, experiments were conducted in which the levels of cystine were increased gradually up to the optimal sulfur amino acid content, with various levels of choline additions. With levels of choline chloride as high as 0.6%, the growth obtained was no better than 4% per day, while substitution of part of the cystine by methionine so that the total methionine content of the diet was 0.55%, gave optimal gains of 6% per day.

Cystine apparently cannot compensate for a methionine deficiency, even if high levels of choline are employed.

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